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(54) Title: REGULATION OF T CELL-MEDIATED IMMUNITY BY TRYPTOPHAN		
(57) Abstract <p>A mechanism of macrophage-induced T cell suppression is the selective elimination of tryptophan and/or increase in one or more tryptophan metabolites within the local macrophage microenvironment. Studies demonstrate that expression of IDO can serve as a marker of suppression of T cell activation, and may play a significant role in allogeneic pregnancy and therefore other types of transplantation, and that inhibitors of IDO can be used to activate T cells and therefore enhance T cell activation when the T cells are suppressed by pregnancy, malignancy or a virus such as HIV. Inhibiting tryptophan degradation (and thereby increasing tryptophan concentration while decreasing tryptophan metabolite concentration), or supplementing tryptophan concentration, can therefore be used in addition to, or in place of, inhibitors of IDO. Similarly, increasing tryptophan degradation (thereby, decreasing tryptophan concentration and increasing tryptophan metabolite concentration), for example, by increasing IDO concentration or IDO activity, can suppress T cells. Although described particularly with reference to IDO regulation, one can instead manipulate local tryptophan concentrations, and/or modulate the activity of the high affinity tryptophan transporter, and/or administer other tryptophan degrading enzymes. Regulation can be further manipulated using cytokines such as macrophage colony stimulating factor, interferon gamma, alone or in combination with antigen or other cytokines.</p>		

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5 REGULATION OF T CELL-MEDIATED IMMUNITY BY TRYPTOPHAN

The United States government has certain rights in this invention by virtue of National Institutes of Health K08 grant to David Munn.

10 This application claims priority to U.S. Serial No. 60/067,610 entitled
"Regulation of T Cell Activation" filed December 5, 1997; U.S. Serial No.
60/080,384 entitled "Regulation of Pregnancy" filed April 1, 1998; and U.S.
Serial No. 60/080,380 entitled "IDO Inhibitors for Use as Antiviral Agents"
filed April 1, 1998, by David Munn and Andrew Mellor.

15 The present invention is generally in the area of regulation of T cell
activation using modulators of the enzyme indoleamine 2,3-dioxygenase
(IDO) which is used by immunosuppressive antigen-presenting cells such as
tissue macrophages and placental trophoblasts to prevent T cells from
activating in response to antigens presented by these cells. Modulation of the
20 enzyme activity can therefore be used to affect pregnancy, infection by
certain viruses such as HIV, and inflammation. More specifically, the
present invention includes altering maternal tolerance of pregnancy using
modulators of the enzyme indoleamine 2,3-dioxygenase (IDO) which is used
by immunosuppressive antigen-presenting cells such as tissue macrophages
25 and placental trophoblasts to prevent T cells from activating in response to
antigens presented by these cells.

Background of the Invention

Traditionally, the "professional" antigen-presenting cells (APCs) of the myeloid lineage, dendritic cells and macrophages, have been viewed primarily as accessory cells, functioning simply to assist T cell activation. Recently, however, it has become clear that myeloid-lineage APCs exert a profound influence on T cells, regulating both the nature of the response (humoral versus cellular immunity) and, in some cases, even whether a response occurs at all (activation versus anergy). This has been the subject of recent reviews by Fearon and Locksley (*Science* 1996;272:50-54) and Trinchieri and Gerosa (*J. Leukocyte Biol.* 1996;59:505-511). Currently, the

5 biology of myeloid-lineage APCs is not well understood. Dendritic cells and
macrophages appear to derive from a common progenitor in the
myelomonocytic lineage (Szabolcs, et al. *Blood* 1996;87:4520-4530), but
their markedly different functional characteristics are determined during a
complex process of hematopoietic differentiation, which continues well after
10 their exit from the bone marrow (Thomas, et al. *Stem Cells* 1996;14:196-
206). Hematopoietic differentiation has traditionally fallen outside the
purview of classical immunology.

Macrophages enter the tissues at the immature stage of circulating
monocytes. Using *in vitro* models, it has been shown that the cytokine
15 milieu which they encounter at this early stage determines the phenotype
which they will subsequently adopt. Under the influence of certain cytokines
(in humans, usually GM-CSF plus IL-4 or TNF), monocytes differentiate *in*
vitro into cells which closely resemble dendritic cells (Mackensen, et al.
Blood 1995;86:2699-2707; Rosenzweig, et al., *Blood* 1996;87:535-544). In
20 the presence of inflammatory cytokines they differentiate into macrophages
activated for antigen presentation and host defense (Munn, *Cancer Res.*
1993;53:260-2613; Morahan, et al. In: Heppner GA, Fulton AM, eds.
Macrophages and Cancer. Boca Raton, FL;CRC Press, 1988:1-16.). In the
absence of inflammatory cytokines, monocytes differentiate under the
25 influence of their lineage-specific growth factor, MCSF, into a type of
macrophage which inhibits, rather than supports, T cell activation (Munn, et
al. *J. Immunol.* 1996;156:523-532).

The adaptive immune system must tailor the T cell repertoire so as
not to respond to self antigens. The classical model (reviewed by Nossal in
30 *Cell* 1994;76:229-239) holds that autoreactive T cell clones are deleted in the
thymus via the process of negative selection (encounter with antigen at the
immature thymocyte stage triggers apoptosis, resulting in clonal deletion).
Although the thymus undoubtedly provides a major site of negative
selection, there are two difficulties with this model. First, it would seem
35 unlikely that every developing T cell could be exposed to every self protein
during its relatively brief transit through the thymus. Second, autoreactive T

5 cells are empirically found in the peripheral blood of normal, healthy hosts (Steinman *Cell* 1995;80:7-10). This suggests that there must exist some additional means of tailoring the T cell repertoire after the T cells have left the thymus, a process now designated peripheral tolerance. Multiple mechanisms have been proposed to contribute to peripheral tolerance (for
10 recent reviews see Steinman L. *Cell* 1995;80:7-10; Mondino, et al. *Proc. Natl. Acad. Sci USA* 1996;93:2245-2252; and Quill H. *J. Immunol.* 1996;156:1325-1327).

Most recent studies support a model in which dendritic cells are the primary physiologic route of antigen presentation to T cells (Thomas 1996).
15 Under normal circumstances, this process is felt to occur only in lymph nodes. Given this exclusive role for dendritic cells in initiating immune responses, tissue macrophages represent a paradox. They are professional APCs, but they are also professional scavengers of all manner of damaged cells and proteins, and hence take up a huge array of self antigens.
20 Moreover, unlike dendritic cells, many of them constitutively express MHC and costimulatory ligands (Azuma, et al. *Nature* 1993;366:76-79) and function as APCs *in vitro* (Unanue, et al. *Science* 1987;236:551-557), implying they are constantly prepared to present antigen.

It is not known how they avoid provoking autoimmune responses.
25 One possibility is that T cells never encounter tissue macrophages. This may indeed be the case for naive T cells, since they are not thought to circulate through tissues (Springer, et al. *Cell* 1994;76:301-314). However, at times of injury and inflammation, many self antigens unavoidably enter the normal antigen-presentation pathway along with legitimate foreign antigens (either
30 because the dendritic cell has no way to discriminate between the two, or due to influx of debris from damaged tissues into the draining lymph nodes (Steinman 1995)).

Certain pathological conditions, such as AIDs (caused by the human immunodeficiency virus, HIV) and latent cytomegaloviral (CMV) infections,
35 are extremely difficult to treat since the macrophages act as reservoirs for the viruses. Even though the cells are infected with virus, they are not

5 recognized as foreign. It is not known why these cells are protected from the host's immune system.

It is therefore an object of the present invention to identify mechanisms by which tissue macrophages regulate T cell activation in order to modulate autoimmune responses to the self-derived antigens which they
10 present, especially in the context of infections with facultative intracellular pathogens, such as HIV and CMV.

It has long been a mystery why a pregnant individual does not reject her fetus as foreign. Many theories have been proposed, and various mechanisms suggested. Being able to understand and control this
15 phenomena would be of benefit both for the development of contraceptives or aborticides, as well as in treatment of some women who are unable to carry a fetus full term. Medawar, 1953, *Symp. Soc. Exp. Biol.* 7, 320-328, pointed out 45 years ago that the mammalian conceptus ought to survive gestation because it carries and expresses paternally-inherited polymorphic
20 genes that provide maternal immune responses when expressed by other tissues. The paradox presented by survival of fetal allografts has not yet been explained in mechanistic terms despite much research on the immunology of mammalian reproduction.

Three factors that might explain the immunological paradox of fetal
25 survival are: (1) anatomic separation of mother and fetus, (2) antigenic immaturity of the fetus and (3) immunologic "inertness" (tolerance) of the mother (Medawar 1953). Recently, attention has focused on the third possibility based on evidence that the entire maternal T cell repertoire is transiently tolerized to paternal MHC class I alloantigens during pregnancy
30 (Tafari, et al., 1995, *Science* 270, 630-633). However, it is not clear how transient tolerance is imposed and maintained in the peripheral T cell repertoire during pregnancy.

It is therefor an object of the present invention to identify mechanisms by which rejection of the fetus by its mother are prevented.

35 It is a further object of the present invention to provide reagents and methods for use thereof for terminating or maintaining pregnancies.

5

Summary of the Invention

A mechanism of macrophage-induced T cell suppression is the selective elimination of tryptophan and/or increase in one or more tryptophan metabolites within the local macrophage microenvironment via simultaneous
10 induction of the enzyme indoleamine 2,3-dioxygenase (IDO) and a tryptophan-selective transport system. Studies demonstrate that expression of IDO can serve as a marker of suppression of T cell activation, and may play a significant role in allogeneic pregnancy and therefore other types of transplantation, and that inhibitors of IDO can be used to activate T cells and
15 therefore enhance T cell activation when the T cells are suppressed by malignancy or a virus such as HIV. Inhibiting tryptophan degradation (and thereby increasing tryptophan concentration while decreasing tryptophan metabolite concentration), or supplementing tryptophan concentration, can therefore be used in addition to, or in place of, inhibitors of IDO. Similarly,
20 increasing tryptophan degradation (thereby, decreasing tryptophan concentration and increasing tryptophan metabolite concentration), for example, by increasing IDO concentration or IDO activity, can suppress T cells. Although described particularly with reference to IDO regulation, one can instead manipulate local tryptophan concentrations, and/or modulate the
25 activity of the high affinity tryptophan transporter, and/or administer other tryptophan degrading enzymes. Regulation can be further manipulated using cytokines such as macrophage colony stimulating factor, interferon gamma, alone or in combination with antigen or other cytokines.

Studies demonstrate that expression of IDO can serve as a marker of
30 suppression of T cell activation, and plays a significant role in allogeneic pregnancy. Inhibitors of IDO can be used to activate T cells and thereby induce rejection of a fetus. Studies show that administration of an inhibitor of IDO, 1-methyl-tryptophan, induces specific and uniform rejection of allogeneic conceptus. Embryo loss is preceded by extensive inflammation,
35 the appearance of mononuclear and neutrophil infiltrates, and degeneration of decidual tissues. Rejection is T cell driven since a single paternally-

5 inherited fetal MHC class I alloantigen provokes embryo loss, and rejection does not occur if maternal lymphocytes are absent when IDO activity is inhibited or the mother does not have functional T cells.

Administration of an inhibitor of IDO, 1-methyltryptophan, can cause rejection of foreign tissue without toxicity. These studies provide a
10 mechanism whereby facultative intracellular pathogens are able to avoid destruction by the host's T cells, even when the macrophages express viral antigens on their surfaces. Inhibition of IDO should cause the host to attack and kill the infected macrophages. In a preferred embodiment for treatment of HIV, the viral load is decreased using standard HIV therapy, usually over
15 a period of three to six months. This is then followed by treatment with an inhibitor of IDO such as 1-methyltryptophan at a dose equivalent to 1 g/kg, for a period of between one day and a few weeks. For treatment of CMV infections, especially before cancer chemotherapy or bone marrow transplant, the patient is treated with an inhibitor of IDO for a period
20 effective for the patient's own T cells to attack and kill any infected macrophages. Malignancies are treated with an IDO inhibitor until the tumor(s) is necrotic or the cancer is in remission.

Brief Description of the Drawings

25 Figure 1 is a graph of thymidine incorporation (cpm) by T cells in the presence of supplemental amino acids. Co-culture conditioned medium is selectively depleted of tryptophan. Human monocytes were allowed to differentiate into macrophages then co-cultured with mitogen-activated T cells (anti-CD3). Fresh PBMC were then suspended in this medium and
30 activated with additional anti-CD3. Replicate cultures were supplemented with individual amino acids to the concentrations normally found in RPMI-1640. Control cultures received fresh medium. Proliferation was measured by 4 hr thymidine incorporation after 72 hrs. Error bars show SD.

Figure 2 is a graph of thymidine incorporation as a function of
35 tryptophan concentration (nM) showing the dose-response relationship to tryptophan. Tryptophan was titrated in co-culture conditioned medium as

5 described in Figure 1., and proliferation of T cells measured at 72 hrs. SD was less than 10% and error bars are omitted for clarity.

Figure 3 is a graph of tryptophan concentration (micromoles) over time in hours showing the elimination kinetics of tryptophan in co-cultures. MCSF-derived macrophages were cultured for 24 hrs with autologous T
10 cells, with or without anti-CD3 mitogen. The medium was replaced with fresh medium, and supernatant from replicate cultures harvested at the times shown. The concentration of tryptophan was determined by the formaldehyde/FeCl₂ fluorescence assay. Error bars (less than 10%) are omitted for clarity.

15 Figure 4A and 4B are graphs showing inhibition of the IDO enzyme abrogates suppression. Co-cultures of MCSF-derived Mφs and T cells with anti-CD3 mitogen were supplemented either with the IDO inhibitor-1-methyl-tryptophan (Figure 4A), or with 10x the normal concentration of tryptophan (Figure 4B). Proliferation was measured after 72 hrs by
20 thymidine incorporation. The level of background proliferation contributed by macrophages is indicated by the dotted line. In Figure 4B, macrophages were seeded at both low density (5×10^4 /well) and high density (2×10^5 /well). In both parcels, all differences discussed as significant were $p < .01$ by ANOVA and Tukey's.

25 Figure 5 is a graph of tryptophan degradation (% total) versus interferon concentration (U/ml) showing that CD40-L acts synergistically with IFN γ to induce IDO activity. MCSF-derived macrophages were activated for 24 hrs with various concentrations of recombinant IFN γ , in the presence or absence of recombinant CD40-L trimer. At the end of the
30 activation period the tryptophan remaining in the cultures was assayed as described for Figure 3. SD was less than 10%.

Figure 6 is a graph of tryptophan degradation (%total) over time (hours) for showing developmental regulation of inducible IDO activity. Monocytes were allowed to differentiate in either MCSF or GMCSF+IL-4
35 for 5 days, then activated by sequential exposure to CD40-L and IFN γ . Following activation, the medium was replaced with fresh medium and the

5 rate of tryptophan catabolism measured over 8 hrs. SD was less than 10%.

Figures 7A and 7B are graphs of tryptophan uptake (pmol/ 10^6 cells) over time (min) showing inducible transport of tryptophan into macrophages. Figure 7A shows uptake of [3 H] tryptophan (200 nM) was measured for the times indicated, then cells were washed onto glass fiber filters and assayed
10 by liquid scintillation counting. The graph compares fresh monocytes, MCSF-derived macrophages (day 7), and MCSF-derived macrophages activated for the preceding 24 hrs with IFN γ . All assays were performed in the presence of 140 mM NaCl. Figure 7B shows sodium-dependent and sodium-independent (140 mM choline chloride) uptake for T cells activated
15 for 72 hrs with anti-CD3, then assayed as in Figure 7A. A logarithmic scale is used to permit comparison.

Figures 8A and 8B are graphs of tryptophan uptake (cpm) in the presence of various competing unlabeled amino acids for dendritic cells (Figure 8A) and MCSF macrophages (Figure 8B) showing sodium-
20 independent transport of tryptophan in MCSF-derived macrophages does not have the characteristics of system L. Monocytes were allowed to differentiate for 5 days in the presence of either GMCSF + IL-4 (dendritic cells, Figure 8A) or MCSF (macrophages, Figures 8B). Sodium-independent tryptophan transport was assayed as described in Figure 7 in the presence of
25 various competing unlabeled amino acids (8 mM each).

Figure 9 is a graph of percent inhibition versus concentration of competitor (tryptophan or phenylalanine) showing that the tryptophan transport system in MCSF-derived macrophages displays a preferential affinity for tryptophan. Macrophages were cultured for 7 days in MCSF and
30 activated during the last 24 hrs with IFN γ . Uptake of [3 H]tryptophan (400 nM) was then assayed in the presence of various concentrations of unlabeled tryptophan or phenylalanine.

Figure 10 are HPLC analyses showing elimination of autoreactive T cells *in vivo* is accompanied by tryptophan degradation. Antigen-transgenic
35 mice received autoreactive T cells by adoptive transfer, and then were assayed for the tryptophan breakdown product kynurinine at a time when the

5 T cells were being actively eliminated (day 3). Trace 1 shows HPLC analysis for kynurinine in control serum prior to adoptive transfer. Trace 3 shows control serum spiked with authentic kynurinine (10 μ M). Trace 2 shows serum from mice on day 3 after adoptive transfer of T cells. The asterisk indicates the kynurinine peak.

10 Figure 11 is a graph of autoreactive TcR-transgenic T cells (1×10^7) over time (days) after adoptive transfer of cells with and without inhibitor.

Detailed Description of the Invention

Roles of IDO and Tryptophan in T Cell Activation

15 As monocytes differentiate into macrophages they can adopt markedly different phenotypes, depending on the cytokine milieu. Macrophages may present antigens in an immunosuppressive, rather than immunostimulatory fashion. Most phenotypes support T cell activation, but one, produced by exclusive exposure to the cytokine macrophage colony-stimulating factor (MCSF), suppresses T cell activation. It is proposed that
20 macrophages discriminate which antigens are "self" based on the presence or absence of inflammation during terminal macrophage differentiation. Inflammatory cytokines such as IFN γ (Munn, et al. 1993; Munn, et al. 1996), IFN α , and IL-4 act dominantly over MCSF to prevent the
25 development of the inhibitory macrophage phenotype. Thus, immunosuppressive macrophages should develop only if they differentiated in normal, uninfamed tissues. In such tissues, the only antigens encountered should be self-derived, and hence responding T cells would be presumptively autoreactive. This is conceptually related to proposed models of self/non-self discrimination such as the model described by Janeway *Immunol. Today*
30 1993;13:11-16, in which antigens in normal tissues are considered self, and only in settings of inflammation or infection are T cells allowed to activate. This model has been subsequently reviewed by Ibrahim in *Immunol. Today* 1995;16:181-186.

35 The data and proposed mechanisms described herein complement these models by suggesting a specific mechanism by which antigens could be

5 presented in normal tissues in an immunosuppressive fashion. The mechanism of this suppression is via induction of the enzyme indoleamine 2,3-dioxygenase (IDO), which selectively degrades the essential amino acid tryptophan. In non-hepatic tissues, the enzyme responsible for the initial rate-limiting step in tryptophan degradation along the kynurinine pathway is
10 indoleamine-2,3-dioxygenase (IDO) (Taylor, et al. *FASEB J.* 1991;5:2516-2522). IDO is a subject of active current interest in the context of infectious disease (Pfefferkorn *Proc. Natl. Acad. Sci. USA* 1984;81:908-912; Gupta *Infect. Immun.* 1994;62:2277-2284; Musso; *Blood* 1994;83:1408-1411; Koide *Infect. Immun.* 1994;62:948-955; Daubener *Immunol.* 1995;86:79-84;
15 Daubener, et al., *Eur. J. Immunol.* 1996;26:487-492; Carlin, et al. *J. Leuk. Biol.* 1989;45:29-34; Nagineni *Infect. Immun.* 1996;64:4188-4196), where it is postulated to reduce the intracellular concentration of tryptophan to the point that facultative intracellular pathogens are unable to replicate (tryptophan is an essential amino acid for all eukaryotic parasites, and for
20 certain simple prokaryotes). Cell lines which have mutations in the IDO gene are unable to control the replication of organisms such as toxoplasma and chlamydia (Thomas *J. Immunol.* 1993; 150:5529-5534). Conversely, pathogen-sensitive cell lines transfected with IDO acquire the ability to inhibit proliferation of these organisms (Gupta *Infect Immun.* 1994;62:2277-
25 2284). IDO may be particularly critical in defense against CNS and ocular toxoplasmosis, since glial and retinal cells show a direct correlation between IDO activity and control of toxoplasma (Daubener *Eur. J. Immunol.* 1996;26:487-492; Nagineni *Infect. Immun.* 1996;64:4188-4196). IDO may also contribute to the pathophysiology of central nervous system infection, autoimmune inflammation, and AIDS dementia, via both local tryptophan
30 starvation and by generation of neurotoxic tryptophan metabolites such as quinolinate (Venkateshan *Proc. Natl. Acad. Sci. USA* 1996;93:1636-1641; Vogelsang, *J. Rheumatol.* 1996;23:850-855; Sardar *Neurosci. Lett.* 1995;187:9-12; Alberati-Giani *J. Neurochem.* 1996;66:996-1004).
35 IDO is induced by several inflammatory mediators, including interferons and LPS, as well as by viral infection (probably indirectly via

5 interferons). The most potent inducer of IDO is IFN γ , due to two interferon-stimulated response elements (ISREs) in the IDO promoter (Konan J. Biol. Chem. 1996;271:19140-19145; Chon J. Biol. Chem. 1996;271:17247-17252). In addition to its effect on intracellular organisms, IDO appears to mediate at least part of the antiproliferative effect of IFN γ on replicating host
10 cells, such as virally infected cells and tumor cells (Aune, et al., 1989 *J. Clin. Invest.* 84, 863-875). Mutant cell lines selected for their ability to grow in the presence of IFN γ were found to have acquired mutations in the IDO gene or its promoter (Feng Proc. Natl. Acad. Sci USA 1989; 86:7144-7148.). Allogeneic tumor cells being rejected by the host immune system *in vivo*
15 upregulate IDO, and this effect is mediated by IFN γ (Takikawa, et al. In: Schwarcz R, ed. Kynurenine and Serotonin Pathways. New York: Plenum Press, 1991:437-444; Yu, et al., *Intl. Immunol.* 1996;8:855-865), although it is not known whether IDO plays a causal role in tumor rejection. Thus, IDO can inhibit proliferation of both intracellular pathogens and host cells.

20 The prior art in the field has viewed IDO solely as a means to reduce the concentration of tryptophan within the IDO-expressing cell (Taylor and Feng, 1991 *FASEB J.* 5:2516-2522). There has been no speculation that IDO could function to suppress proliferation of neighboring cells, and specifically no suggestion that it could be used to suppress T cell activation. Based on
25 the *in vitro* studies described herein, the hypothesis was formulated that cells expressing IDO would be able to suppress T cell activation *in vivo*. From this hypothesis the testable prediction was formulated that inhibition of IDO enzyme activity with compounds such as 1-methyl-tryptophan would allow enhanced T activation *in vivo*. Review of the existing literature suggested
30 that IDO activity was likely to be present in macrophage-like and dendritic-like cells in organs of the immune system (Moffett, et al., 1994 *Cell Tissue Res.* 278:461-469). Therefore, to test the hypothesis that IDO-expressing cells in the immune system suppressed autoreactive T cells *in vivo*, adoptive transfer studies were performed in which T cells which were transgenic for a
35 defined T cell receptor were injected into recipient mice made transgenic for the cognate target antigen. This model of autoimmune T cell activation has

5 been previously described by Tarazona, et al., 1996 *Int. Immunol.* 8:351-358. As shown in Figure 10, the injection of autoreactive T cells resulted in induction of IDO (measured as elevated kynurenine in serum). When IDO activity was inhibited in recipient animals by administration of 1-methyl-tryptophan, the activation of the autoreactive T cells was markedly enhanced
10 (2- to 3-fold over control, as shown by Figure 11). These data thus supported the hypothesis that there existed a population of IDO-expressing cells which suppressed autoreactive T cells *in vitro*.

The *in vitro* data presented herein were derived using human monocyte-derived macrophages. For convenience, the term "macrophage" is
15 therefore used to describe any IDO-expressing cell which suppresses T cell activation. However, *in vivo* this cell could be a macrophage, dendritic cell, or other cell type which expresses IDO and suppresses T cells.

As determined in the studies described herein, macrophages are primed during differentiation in the presence of MCSF to undergo a massive
20 induction of IDO activity in response to a synergistic combination of signals displayed during early T cell activation (IFN γ plus CD40 ligand). In addition, these macrophages markedly increase their rate of tryptophan uptake, via induction of a previously unidentified high-affinity transport pathway. Macrophages which have differentiated under the influence of
25 macrophage colony-stimulating factor (MCSF) inhibit attempted T cell activation via super-induction of the antimicrobial host-defense enzyme indoleamine 2,3-dioxygenase, which selectively degrades the essential amino acid tryptophan. Simultaneously, these macrophages also markedly increase tryptophan uptake via induction of a novel high-affinity transport pathway.
30 The combination of these two mechanisms allows macrophages of this phenotype to effectively deplete tryptophan from the local microenvironment, thus preventing T cell activation.

Nutrient depletion is an evolutionarily ancient strategy, and as the utility of L-asparaginase against leukemia attests it remains an effective one.
35 The best studied example of this type of mechanism in humans is chelation of iron, an essential micronutrient for all dividing cells. Microbes employ

5 this strategy against each other (deferroxamine is an example), and so do
macrophages via the secretion of the iron-chelating protein lactoferrin
(Baynes *Adv. Exp. Med. Biol.* 1994;357:133-141). Macrophages use iron
depletion as a cytostatic mechanism against tumor cells and other dividing
cells (reviewed in Weiss, et al. *Immunology Today* 1995;16:495-500), and
10 iron is rapidly sequestered from serum and tissues by macrophages in
response to infection and inflammation. Another example of regulation by
nutrient depletion is the ability of macrophages to alter the intracellular thiol
pool in T cells via degradation of cysteine, resulting in thiol-mediated redox
regulation of T cell activation (Gmunder, et al. *Cell. Immunol.* 1990;129:32-
15 46; Iwata, et al., *J. Immunol.* 1994;152:5633-5642). Thus, tryptophan
degradation is not a unique phenomenon, but rather belongs to a class of
mechanisms whereby proliferation is inhibited by local depletion of a factor
essential for growth. Like macrophages themselves (Ottaviani, et al.
Immunol. Today 1997;18:169-174), the IDO gene is evolutionarily ancient.
20 An homologous gene exists in lower invertebrates, and the intron-exon
structure has been highly conserved throughout 600 million years of
evolution (Suzuki, et al. *Biochem. Biophys. Acta* 1996;1308:41-48). This
suggests that tryptophan degradation is an effective and important strategy.

A key assumption implicit in the hypothesis is that macrophages *in*
25 *vivo* are able to create a local microenvironment in which the tryptophan
concentration is very low, despite the availability of tryptophan in the
bloodstream. In theory, this should be possible because the rate of
tryptophan delivery is limited by diffusion through the interstitial space.
Burke, et al, have directly tested this assumption by measuring tryptophan
30 concentrations in tumor xenografts which had been induced to express IDO
by IFN γ (Burke, et al. *Int. J. Cancer* 1995;60:115-122). These authors found
an approximately 80% reduction in tumor tryptophan in IFN γ -treated
animals compared to controls, indicating that the rate of local degradation
did indeed exceed the rate of delivery.

35 The IDO enzyme is known from published literature to be expressed
in syncytiotrophoblast cells of the human placenta (Kamimura, et al., 1991

5 *Acta. Med. Okayama* 45:135-139). Syncytiotrophoblasts are fetal-derived cells which comprise the zone of contact between fetal tissues and the maternal bloodstream (and hence contact with the maternal immune system). The presence of IDO in placenta had been confusing to researchers in the field, since the placenta supplies nutrients to the fetus and it seemed
10 paradoxical that it should actively degrade an essential nutrient such as tryptophan. Based on the *in vitro* and *in vivo* studies described herein, the hypothesis was formulated that the role of IDO in the placenta was to suppress T cell responses against the genetically "foreign" fetus. The survival of the mammalian fetus has long been a paradox in the field of
15 immunology (Medawar, 1953, *Symp. Soc. Exp. Biol.* 7:320-328), since half of the fetus's genes are paternally inherited, and hence should provoke an allogeneic rejection response from the maternal immune system. Based on the data herein, it was predicted that if IDO activity were inhibited in placenta the fetus would be unable to protect itself against maternal T cells,
20 and would be rejected. As shown in Table 1, inhibition of IDO with 1-methyl-tryptophan resulted in prompt, T cell-mediated rejection of all allogeneic fetuses. There was no effect of inhibitor on genetically identical (syngeneic) control fetuses, demonstrating that the inhibitor itself was not toxic. This provided definitive support for the ability of IDO to suppress T
25 cell responses *in vivo*. It also showed that IDO-expressing cells other than macrophages (in this case, trophoblasts) were able to suppress T cell responses, indicating that IDO is a broadly applicable mechanism of immune suppression. Specifically, based on the data herein, it is believed that antigen-presenting cells (such as macrophages and dendritic cells)
30 throughout the immune system use IDO to suppress unwanted T cell responses (e.g., to self antigens).

The fetus represents a dramatic example of a solid-tissue "allograft" which is tolerated by the maternal immune system throughout gestation. Prior to the discoveries described herein, it was uncertain whether the fetus
35 presented the transplantation antigens needed to provoke rejection, and, if it did, why the maternal immune system chose to tolerate these antigens. The

5 data in the examples reveal that the fetus does indeed present the relevant transplantation antigens (a difference in even a single MHC gene was sufficient to provoke rejection when IDO was inhibited, see Table 3), and that the maternal immune system did not "choose" to tolerate the fetus, but rather was actively prevented from mounting a response by placental
10 expression of IDO. Taken together, these observations lead to two categories of clinical applications:

Category 1: Since IDO and tryptophan catabolism represent a new mechanism of T cell suppression, the introduction of the IDO gene by transgenesis, the use of pharmacologic inducers of IDO enzyme activity, or
15 the direct introduction of IDO or IDO-like enzymatic activity as purified proteins into relevant sites, can all be used for local immunosuppression.

Category 2: Since inhibition of IDO is able to restore T cell responses which would otherwise be suppressed (as in the pregnancy model described herein), pharmacologic inhibitors of the IDO enzyme could be
20 used to restore desirable T cell responses which are normally suppressed by IDO.

With regard to Category 1 (use of IDO as an immunosuppressant), the most direct application is to simply inject or otherwise deliver the purified IDO enzyme as a local immunosuppressive agent. However,
25 because IDO contains a heme prosthetic group and requires a complex regenerating system in order to avoid auto-oxidation in vitro (Sono, et al., 1980. *J. Biol. Chem.* 255:1339-1345), it may be difficult to employ the purified enzyme directly as a pharmacologic agent. A strategy to surmount this difficulty is to use purified bacterial enzymes such as indolyl-3-alkane
30 alpha-hydroxylase, which have IDO-like tryptophan-degrading activity and which have been shown to function in purified form (U.S. Patent Nos. 5,723,325 and 5,244,807 to Murtfeldt, et al).

A second approach is to confer IDO enzymatic activity in a tissue-specific manner by the use of IDO as a transgene under an inducible or
35 constitutive promoter. There is currently significant interest in developing genetically modified animals (e.g., swine) to serve as donors for solid organ

5 xenotransplantation (Saadi and Platt 1998 *Life Sci.* 62:265-287). Based on the data herein demonstrating the role of IDO in preventing rejection of the allogeneic fetal "allograft", the use of IDO as a transgene (either as a germline transgene in genetically modified animals, or delivered by transfection into human organs) can be applied to xenogeneic and allogeneic
10 human organ transplantation.

Likewise, there exists a body of literature supporting the use of genetically modified cells to convey immunosuppressive genes into specific anatomic sites of inflammation, e.g., into the synovial space in arthritic joints (Jorgensen and Gay 1998. *Immunol. Today* 19:387-391), for purposes of
15 local immunosuppression, or systemically for purposes of inducing tolerance to subsequent allograft transplantation. These would be additional applications of IDO used as a transgene.

Finally, there is a well-established but poorly understood correlation between tryptophan metabolism and autoimmune disorders, as described
20 further in Example 8. Based on the data herein, it is proposed that IDO suppresses autoreactive T cell activation by degrading tryptophan in local microenvironments. From this it follows that elevated systemic tryptophan levels would make it more difficult for macrophages to deplete tryptophan locally and hence suppress autoreactive T cells; whereas lowered systemic
25 tryptophan levels would favor suppression of autoreactive T cells. Therefore, methods to lower systemic tryptophan levels by pharmacologic induction of IDO, administration of tryptophan-degrading enzymes, or *ex vivo* depletion of plasma tryptophan by tryptophan-degrading enzymes, could be used in the treatment of autoimmune disorders. Examples of
30 suitable enzymes for lowering tryptophan such as indolyl-3-alkane alpha-hydroxylase, and means for *ex vivo* treatment of plasma to lower tryptophan levels which are abnormally elevated are described in U.S. Patent Nos. 5,723,325 and 5,244,807 to Murtfeldt, et al.

Inhibitors of the IDO enzyme (such as 1-methyl-DL-tryptophan, β -
35 (3-benzofuranyl)-DL-alanine, 6-nitro-L-tryptophan and β -[3-benzo(b)thienyl]-DL-alanine), or the high affinity tryptophan transporter can

5 be used to simulate T cell mediated immune responses where these would normally be suppressed by IDO. Applications include using these agents (systemically or locally administered) to an individual to terminate pregnancy. Other applications include transfection of the IDO gene (under an inducible promoter) into tissues and cells prior to or at the time of
10 pregnancy, to reduce the possibility of immune-mediated rejection of the fetus. Screening of tryptophan levels and/or IDO expression can be used as indicators of T cell activation or suppression and therefore predictors of miscarriage or spontaneous abortion.

Based on these studies and the underlying mechanism, it is possible
15 to terminate, or help to sustain in some individuals, pregnancy. To terminate the pregnancy one administers an effective amount of an inhibitor of IDO, or transport of tryptophan by the high affinity tryptophan transporter. For example, an inhibitor such as 1-methyltryptophan at a dosage of 1 g/kg is effective to cause rejection and killing by macrophages. The dosage may be
20 administered one or more times, as required, to yield the desired effect.

Compounds which Modulate Tryptophan or Tryptophan Metabolite

A number of techniques are known for obtaining compounds which can be used to modulate tryptophan or tryptophan metabolite levels. The
25 active compounds can be divided into the following categories:

- (1) IDO and other enzymes whos activity can be modulated to alter tryptophan degradation
- (2) The high affinity sodium-independent extremely selective tryptophan transporter and other transporters of tryptophan which can
30 increase or decrease typtophan transport into the cell

IDO is well characterized, as discussed above. Bacterial enzymes, also discussed herein, can also be used to degrade tryptophan. Tryptophan transporter are described herein and in the literature. Although the following description is primarily directed towards modulation of IDO activity, the
35 descriptions are equally applicable for modulators of the high affinity tryptophan transporter, which can be regulated in the same manner as other

5 transporter molecules, by turning on or off transport by completely blocking access to the transporter, or by partially blocking access, for example, by binding of the transporter with a molecule which competitively binds with tryptophan, or which has a higher binding affinity or lower dissociation constant than tryptophan. IDO is an intracellular enzyme. In order for it to
10 affect the extracellular level of tryptophan its substrate must be transported across the macrophage cell membrane. There are many known amino acid transport systems in mammalian cells, a number of which accept tryptophan. However, none of these are specific for tryptophan, so it must compete with various other amino acids for uptake. To date, no preferential tryptophan
15 transporter has been described in mammalian cells. Data suggests that there may exist an IFN γ -inducible, high-affinity, tryptophan-selective uptake system in MCSF-derived macrophages. It is sodium-independent, placing it in a limited category. The known sodium-independent systems are summarized in Kakuda *J. Exp. Biol.* 1994;196:93-108; McGivan *Biochem. J.*
20 1994;299:321-324. In some ways the macrophage transport system resembles system L. However, in cross-competition studies, the tryptophan uptake system in macrophages shows a 10- to 100-fold higher affinity for tryptophan than for the next best substrate, phenylalanine, which does not correspond to any of the known systems. System T, which also accepts
25 tryptophan, has a much lower affinity than the system observed in macrophages (Zhou *J. Biol. Chem.* 1990;265:17000-17004). I

Assays for testing compounds for useful activity can be based solely on interaction with IDO or enzymes or the transporters involved in tryptophan metabolism ("the enzymes"), or alternatively, the assays can be
30 based on interaction with the gene sequence encoding the enzymes. For example, antisense which binds to the regulatory sequences, and/or to the protein encoding sequences can be synthesized using standard oligonucleotide synthetic chemistry. The antisense can be stabilized for pharmaceutical use using standard methodology (encapsulation in a liposome
35 or microsphere; introduction of modified nucleotides that are resistant to degradation or groups which increase resistance to endonucleases, such as

5 phosphorothiodates and methylation), then screened initially for alteration of enzyme activity in transfected or naturally occurring cells which express the enzyme, then *in vivo* in laboratory animals. Typically, the antisense would inhibit expression. However, sequences which block those sequences which "turn off" synthesis can also be targeted

10 Compounds which inhibit IDO are known, as demonstrated by the examples, although not previously described for use as immunomodulators. Additional compounds which are highly selective can be obtained as follows.

Random generation of enzyme or enzyme encoding sequence binding molecules.

15 Molecules with a given function, catalytic or ligand-binding, can be selected for from a complex mixture of random molecules in what has been referred to as "in vitro genetics" (Szostak, TIBS 19:89, 1992). One synthesizes a large pool of molecules bearing random and defined sequences and subjects that complex mixture, for example, approximately 10^{15}
20 individual sequences in 100 μ g of a 100 nucleotide RNA, to some selection and enrichment process. For example, by repeated cycles of affinity chromatography and PCR amplification of the molecules bound to the ligand on the column, Ellington and Szostak (1990) estimated that 1 in 10^{10} RNA molecules folded in such a way as to bind a given ligand. DNA molecules
25 with such ligand-binding behavior have been isolated (Ellington and Szostak, 1992; Bock et al, 1992).

Computer assisted drug design

Computer modeling technology allows visualization of the three-dimensional atomic structure of a selected molecule and the rational design
30 of new compounds that will interact with the molecule. The three-dimensional construct typically depends on data from x-ray crystallographic analyses or NMR imaging of the selected molecule. The molecular dynamics require force field data. The computer graphics systems enable prediction of how a new compound will link to the target molecule and allow
35 experimental manipulation of the structures of the compound and target molecule to perfect binding specificity. Prediction of what the molecule-compound interaction will be when small changes are made in one or both

- 5 requires molecular mechanics software and computationally intensive computers, usually coupled with user-friendly, menu-driven interfaces between the molecular design program and the user.

Examples of molecular modelling systems are the CHARMM and QUANTA programs, Polygen Corporation, Waltham, MA. CHARMM
10 performs the energy minimization and molecular dynamics functions. QUANTA performs the construction, graphic modelling and analysis of molecular structure. QUANTA allows interactive construction, modification, visualization, and analysis of the behavior of molecules with each other.

- 15 A number of articles review computer modeling of drugs interactive with specific proteins, such as Rotivinen, et al., 1988 Acta Pharmaceutica Fennica 97, 159-166; Ripka, New Scientist 54-57 (June 16, 1988); McKinaly and Rossmann, 1989 Annu. Rev. Pharmacol. Toxicol. 29, 111-122; Perry and Davies, QSAR: Quantitative Structure-Activity Relationships in Drug
20 Design pp. 189-193 (Alan R. Liss, Inc. 1989); Lewis and Dean, 1989 Proc. R. Soc. Lond. 236, 125-140 and 141-162; and, with respect to a model enzyme for nucleic acid components, Askew, et al., 1989 J. Am. Chem. Soc. 111, 1082-1090. Other computer programs that screen and graphically depict chemicals are available from companies such as BioDesign, Inc.,
25 Pasadena, CA., Allelix, Inc, Mississauga, Ontario, Canada, and Hypercube, Inc., Cambridge, Ontario. Although these are primarily designed for application to drugs specific to particular proteins, they can be adapted to design of drugs specific to regions of DNA or RNA, once that region is identified.

- 30 Although described above with reference to design and generation of compounds which could alter binding, one could also screen libraries of known compounds, including natural products or synthetic chemicals, and biologically active materials, including proteins, for compounds which are inhibitors or activators.

35 *Generation of nucleic acid regulators*

Nucleic acid molecules containing the 5' regulatory sequences of the

5 enzyme genes can be used to regulate or inhibit gene expression *in vivo*.
Vectors, including both plasmid and eukaryotic viral vectors, may be used to
express a particular recombinant 5' flanking region-gene construct in cells
depending on the preference and judgment of the skilled practitioner (see,
e.g., Sambrook et al., Chapter 16). Furthermore, a number of viral and
10 nonviral vectors are being developed that enable the introduction of nucleic
acid sequences *in vivo* (see, e.g., Mulligan, 1993 Science, 260, 926-932;
United States Patent No. 4,980,286; United States Patent No. 4,868,116;
incorporated herein by reference). Nucleic acid can be encapsulated in
cationic liposomes which can be injected intravenously into a mammal (Zhu
15 et al., 1993 Science 261, 209-211).

The 5' flanking sequences of the enzyme gene can also be used to
inhibit the expression of the enzyme. For example, an antisense RNA of all
or a portion of the 5' flanking region of the enzyme gene can be used to
inhibit expression of the enzyme *in vivo*. Expression vectors (e.g., retroviral
20 expression vectors) are already available in the art which can be used to
generate an antisense RNA of a selected DNA sequence which is expressed
in a cell (see, e.g., U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286).
Accordingly, DNA containing all or a portion of the sequence of the 5'
flanking region of the enzyme gene can be inserted into an appropriate
25 expression vector so that upon passage into the cell, the transcription of the
inserted DNA yields an antisense RNA that is complementary to the mRNA
transcript of the enzyme gene normally found in the cell. This antisense
RNA transcript of the inserted DNA can then base-pair with the normal
mRNA transcript found in the cell and thereby prevent the mRNA from
30 being translated. It is of course necessary to select sequences of the 5'
flanking region that are downstream from the transcriptional start sites for
the enzyme gene to ensure that the antisense RNA contains complementary
sequences present on the mRNA.

Antisense RNA can be generated *in vitro* also, and then inserted into
35 cells. Oligonucleotides can be synthesized on an automated synthesizer
(e.g., Model 8700 automated synthesizer of Milligen-Bioscience, Burlington,

- 5 MA or ABI Model 380B). In addition, antisense deoxyoligonucleotides have been shown to be effective in inhibiting gene transcription and viral replication (see e.g., Zamecnik et al., 1978 Proc. Natl. Acad. Sci. USA 75, 280-284; Zamecnik et al., 1986 Proc. Natl. Acad. Sci., 83, 4143-4146; Wickstrom et al., 1988 Proc. Natl. Acad. Sci. USA 85, 1028-1032; Crooke,
- 10 1993 FASEB J. 7, 533-539. Inhibition of expression of a gene by antisense oligonucleotides is possible if the antisense oligonucleotides contain modified nucleotides (see, e.g., Offensperger et. al., 1993 EMBO J. 12, 1257-1262 (*in vivo* inhibition of duck hepatitis B viral replication and gene expression by antisense phosphorothioate oligodeoxynucleotides);
- 15 Rosenberg et al., PCT WO 93/01286 (synthesis of sulfurthioate oligonucleotides); Agrawal et al., 1988 Proc. Natl. Acad. Sci. USA 85, 7079-7083 (synthesis of antisense oligonucleoside phosphoramidates and phosphorothioates to inhibit replication of human immunodeficiency virus-1); Sarin et al., 1989 Proc. Natl. Acad. Sci. USA 85, 7448-7794 (synthesis of
- 20 antisense methylphosphonate oligonucleotides); Shaw et al., 1991 Nucleic Acids Res 19, 747-750 (synthesis of 3' exonuclease-resistant oligonucleotides containing 3' terminal phosphoroamidate modifications); incorporated herein by reference).

The sequences of the 5' flanking region of enzyme gene can also be

25 used in triple helix (triplex) gene therapy. Oligonucleotides complementary to gene promoter sequences on one of the strands of the DNA have been shown to bind promoter and regulatory sequences to form local triple nucleic acid helices which block transcription of the gene (see, e.g., 1989 Maher et al., Science 245, 725-730; Orson et al., 1991 Nucl. Acids Res. 19, 3435-

30 3441; Postal et al., 1991 Proc. Natl. Acad. Sci. USA 88, 8227-8231; Cooney et al., 1988 Science 241, 456-459; Young et al., 1991 Proc. Natl. Acad. Sci. USA 88, 10023-10026; Duval-Valentin et al., 1992 Proc. Natl. Acad. Sci. USA 89, 504-508; 1992 Blume et al., Nucl. Acids Res. 20, 1777-1784; 1992 Grigoriev et al., J. Biol. Chem. 267, 3389-3395.

35 Both theoretical calculations and empirical findings have been reported which provide guidance for the design of oligonucleotides for use in

5 oligonucleotide-directed triple helix formation to inhibit gene expression. For example, oligonucleotides should generally be greater than 14 nucleotides in length to ensure target sequence specificity (see, e.g., Maher et al., (1989); Grigoriev et al., (1992)). Also, many cells avidly take up oligonucleotides that are less than 50 nucleotides in length (see e.g., Orson et
10 al., (1991); Holt et al., 1988 Mol. Cell. Biol. 8, 963-973; Wickstrom et al., 1988 Proc. Natl. Acad. Sci. USA 85, 1028-1032). To reduce susceptibility to intracellular degradation, for example by 3' exonucleases, a free amine can be introduced to a 3' terminal hydroxyl group of oligonucleotides without loss of sequence binding specificity (Orson et al., 1991). Furthermore, more
15 stable triplexes are formed if any cytosines that may be present in the oligonucleotide are methylated, and also if an intercalating agent, such as an acridine derivative, is covalently attached to a 5' terminal phosphate (e.g., via a pentamethylene bridge); again without loss of sequence specificity (Maher et al., (1989); Grigoriev et al., (1992)).

20 Methods to produce or synthesize oligonucleotides are well known in the art. Such methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see e.g., Sambrook et al., Chapters 5, 6) to purely synthetic methods, for example, by the cyanoethyl phosphoramidite method using a Milligen or Beckman System 1Plus DNA synthesizer (see
25 also, Ikuta et al., in Ann. Rev. Biochem. 1984 53, 323-356 (phosphotriester and phosphite-triester methods); Narang et al., in Methods Enzymol., 65, 610-620 (1980) (phosphotriester method).

Preparation of Enzyme Fragments

30 Compounds which are effective for blocking binding of the enzyme can also consist of fragments of the enzymes, expressed recombinantly and cleaved by enzymatic digest or expressed from a sequence encoding a peptide of less than the full length enzyme. It is a routine matter to make appropriate enzyme fragments, and test for inhibition of activity of the enzyme in the presence of the fragments. The preferred fragments are of
35 human origin, in order to minimize potential immunological response. The peptides can be as short as five to eight amino acids in length and are easily

5 prepared by standard techniques. They can also be modified to increase *in vivo* half-life, by chemical modification of the amino acids or by attachment to a carrier molecule or inert substrate. The peptides can also be conjugated to a carrier protein such as keyhole limpet hemocyanin by its N-terminal cysteine by standard procedures such as the commercial Imject kit from
10 Pierce Chemicals or expressed as a fusion protein, which may have increased efficacy. As noted above, the peptides can be prepared by proteolytic cleavage of the enzymes, or, preferably, by synthetic means. These methods are known to those skilled in the art. An example is the solid phase synthesis described by J. Merrifield, 1964 J. Am. Chem. Soc. 85, 2149, used in U.S.
15 Patent No. 4,792,525, and described in U.S. Patent No. 4,244,946, wherein a protected alpha-amino acid is coupled to a suitable resin, to initiate synthesis of a peptide starting from the C-terminus of the peptide. Other methods of synthesis are described in U.S. Patent No. 4,305,872 and 4,316,891. These methods can be used to synthesize peptides having identical sequence to the
20 enzymes described herein, or substitutions or additions of amino acids, which can be screened for activity as described above.

The peptide can also be administered as a pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric
25 acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases
30 such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

Peptides containing cyclopropyl amino acids, or amino acids derivatized in a similar fashion, can also be used. These peptides retain their original activity but have increased half-lives *in vivo*. Methods known for modifying amino acids, and their use, are known to those skilled in the art,
35 for example, as described in U.S. Patent No. 4,629,784 to Stammer.

5 *Pharmaceutical Compositions*

Compounds which alter enzyme activity and/or tryptophan transport are preferably administered in a pharmaceutically acceptable vehicle. Suitable pharmaceutical vehicles are known to those skilled in the art. For parenteral administration, the compound will usually be dissolved or
10 suspended in sterile water or saline. For enteral administration, the compound will typically be administered in a tablet or capsule, which may be enteric coated, or in a formulation for controlled or sustained release. Many suitable formulations are known, including polymeric or protein microparticles encapsulating drug to be released, ointments, gels, or
15 solutions which can be used topically or locally to administer drug, and even patches, which provide controlled release over a prolonged period of time. These can also take the form of implants. For example prevention of pregnancy could be obtained by administration intravaginally of a compound increasing tryptophan, for example, by inhibiting IDO, thereby resulting in
20 killing of the sperm or fertilized egg almost immediately.

Generation of Transgenic Animals for Screening

With the knowledge of the cDNA encoding the enzyme and regulatory sequences regulating expression thereof, it is possible to generate transgenic animals, especially rodents, for testing the compounds which can
25 alter enzyme expression, translation or function in a desired manner.

There are basically two types of animals which are useful: those not expressing functional enzyme, and those which overexpress enzyme, either in those tissues which already express the protein or in those tissues where only low levels are naturally expressed. The animals in the first group are
30 preferably made using techniques that result in "knocking out" of the gene for enzyme, although in the preferred case this will be incomplete, either only in certain tissues, or only to a reduced amount. These animals are preferably made using a construct that includes complementary nucleotide sequence to the enzyme gene, but does not encode functional enzyme, and is
35 most preferably used with embryonic stem cells to create chimeras. Animals which are heterozygous for the defective gene can also be obtained by

5 breeding a homozygote normal with an animal which is defective in production of enzyme.

The animals in the second group are preferably made using a construct that includes an unregulated promoter or one which is modified to increase expression as compared with the native promoter. The regulatory
10 sequences for the enzyme gene can be obtained using standard techniques based on screening of an appropriate library with the cDNA encoding enzyme. These animals are most preferably made using standard microinjection techniques.

These manipulations are performed by insertion of cDNA or genomic
15 DNA into the embryo using microinjection or other techniques known to those skilled in the art such as electroporation, as described below. The DNA is selected on the basis of the purpose for which it is intended: to inactivate the gene encoding an enzyme or to overexpress or express the gene encoding enzyme. The enzyme encoding gene can be modified by
20 homologous recombination with a DNA for a defective enzyme, such as one containing within the coding sequence an antibiotic marker, which can then be used for selection purposes.

Animals suitable for transgenic experiments can be obtained from standard commercial sources. These include animals such as mice and rats
25 for testing of genetic manipulation procedures, as well as larger animals such as pigs, cows, sheep, goats, and other animals that have been genetically engineered using techniques known to those skilled in the art. These techniques are briefly summarized below based principally on manipulation of mice and rats. The procedures for manipulation of the embryo and for
30 microinjection of DNA are described in detail in Hogan et al. Manipulating the mouse embryo, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1986), the teachings of which are incorporated herein. These techniques are readily applicable to embryos of other animal species, and, although the success rate is lower, it is considered to be a routine practice to those skilled
35 in this art. Methods for the culturing of ES cells and the subsequent production of transgenic animals, the introduction of DNA into ES cells by a

5 variety of methods such as electroporation, calcium phosphate/DNA precipitation, and direct injection are described in detail in Teratocarcinomas and embryonic stem cells, a practical approach, ed. E.J. Robertson, (IRL Press 1987), the teachings of which are incorporated herein. Selection of the desired clone of transgene-containing ES cells is accomplished through one
10 of several means. In cases involving sequence specific gene integration, a nucleic acid sequence for recombination with the enzyme gene or sequences for controlling expression thereof is co-precipitated with a gene encoding a marker such as neomycin resistance. Transfection is carried out by one of several methods described in detail in Lovell-Badge, in Teratocarcinomas and embryonic stem cells, a practical approach, ed. E.J. Robertson, (IRL
15 Press 1987) or in Potter et al Proc. Natl. Acad. Sci. USA 81, 7161 (1984). Calcium phosphate/DNA precipitation, direct injection, and electroporation are the preferred methods. DNA molecules introduced into ES cells can also be integrated into the chromosome through the process of homologous
20 recombination, described by Capecchi, (1989).

Once the transgenic animals are identified, lines are established by conventional breeding and used as the donors for tissue removal and implantation using standard techniques for implantation into humans.

Therapeutic Applications

25 The examples described herein demonstrate that tryptophan depletion and/or catabolism is the mechanism by which macrophages suppress T cell activation, using *in vitro* human and *in vivo* transgenic mouse models; defines the developmental regulation of the IDO gene during hematopoietic differentiation of macrophages and dendritic cells; and defines the functional
30 characteristics and developmental regulation of the tryptophan transport pathway in immunosuppressive macrophages. These studies define a new and previously unsuspected role for tryptophan metabolism to immune regulation.

Pharmaceutical compositions based on these discoveries rely on
35 either increasing tryptophan concentrations (and thereby decreasing tryptophan metabolite concentrations) or decreasing tryptophan

5 concentrations (and thereby increasing tryptophan metabolite concentrations). Inhibitors of IDO increase tryptophan concentrations (and thereby decrease tryptophan metabolite concentrations), increasing T cell activation. IDO decreases tryptophan concentrations (and thereby increases tryptophan metabolite concentrations), decreasing T cell activation.

10 Inhibitors of the IDO enzyme, such as 1-methyl-DL-tryptophan, β -(3-benzofuranyl)-DL-alanine, β -[3-benzo(b)thienyl]-DL-alanine, and 6-nitro-L-tryptophan, can be used to simulate T cell mediated immune responses where these would normally be suppressed by IDO. Inhibitors of other enzymes involved in tryptophan metabolism or degradation can also be used.

15 Applications include using these agents (systemically administered) as vaccine adjuvants to increase the immunogenicity of administered antigens; using the agents for immunotherapy of malignancy, including as an adjuvant for therapeutic tumor-cell vaccines, and as an adjunct to cytokine-based immunotherapy of malignancy; and using these agents to help reverse the
20 immunosuppressed state found in AIDS since IDO is known to be induced during HIV infection; co-administering IDO inhibitors with HIV vaccine to enhance the efficacy of HIV vaccines since IDO is induced in macrophage-lineage cells by HIV infection; and generally for any setting in which IDO mediates unwanted suppression of a desired immune response.

25 Alternatively, recombinant IDO can be used as a means of systemic or local T cell suppression. Applications include transfection of the IDO gene (under an inducible promoter) into tissues and cells prior to allotransplantation, in order to allow them to protect themselves from rejection by the host, for example, by retroviral or other transfer of the IDO
30 gene into allogeneic solid-organ grafts (kidney, etc.); by transgenic expression of the gene in porcine or other animals designed as donors for xeno-transplantation, to prevent rejection; or by retroviral or other transfer into cell preparations intended for transplantation (eg, pancreatic islet cells for therapy of diabetes mellitus).

35 Applications also include using these agents (systemically or locally administered) to an individual to terminate pregnancy. Other applications

5 include transfection of the IDO gene (under an inducible promoter) into tissues and cells prior to or at the time of pregnancy, to reduce the possibility of immune-mediated rejection of the fetus. To terminate the pregnancy one administers an effective amount of an inhibitor of IDO, or transport of
10 inhibitor such as 1-methyltryptophan at a dosage of 1 g/kg is effective to cause rejection and killing by macrophages. The dosage may be administered one or more times, as required, to yield the desired effect.

Screening of tryptophan levels and/or IDO expression can be used as indicators of T cell activation or suppression. Screening of tryptophan levels
15 and/or IDO expression can be used as indicators of T cell activation or suppression and therefore predictors of miscarriage or spontaneous abortion. Testing for IDO expression tumor biopsies can be used to determine the suitability of the cancer for IDO-targeted therapy.

The present invention will be further understood by reference to the
20 following non-limiting examples.

Example 1: Macrophage-induced suppression.

As described in Munn, et al. J. Immunol. 1996;156:523-532, a number of possible mechanisms for macrophage-induced suppression were initially examined. None of those tested, including the fas/fas-L system,
25 nitric oxide, free radicals, prostaglandins, and inhibitory cytokines, could account for the phenomenon. Studies demonstrated the following regarding macrophage inhibition of T cell proliferation:

- (1) MCSF-derived macrophages inhibit the mitogen-induced proliferation of T cells, and this is a dominant effect -- i.e., it is not
30 rescued by adding non-inhibitory APCs.
- (2) The T cells initiate early activation, as assessed by activation-related G1 phase genes (IL-2 receptor, cdc2, cyclin A), but arrest prior to the first G1/S boundary. This arrest is not a stable condition, and most of the lymphocytes progressively die off.
- 35 (3) The suppressive effect was developmentally regulated by MCSF and IFN γ . It was not present in fresh monocytes, emerged progressively

- 5 over 4-5 days of differentiation in MCSF, and was prevented if the monocytes were exposed to IFN γ (even transiently) prior to differentiation. IFN γ has a dual action in macrophage differentiation. If monocytes are exposed to IFN γ *prior* to differentiation it functions as a developmental regulator, and the monocytes will not
- 10 subsequently acquire the suppressive phenotype - even in the presence of MCSF. However, if macrophages are exposed to IFN γ *after* differentiation, it causes activation but does not alter the MCSF-induced phenotype. Thus, the nature of the response to IFN γ is determined by the developmental state of the macrophage.
- 15 (4) There was no evidence for participation of nitric oxide, reactive oxygen species, TNF, TGF β , prostaglandins, IL-1R antagonist, or IL-10. The mechanism of inhibition appeared contact dependent. Inhibition could not be reproduced by conditional medium (up to 50% v/v), and was abrogated when T cells were separated from
- 20 macrophages by a semipermeable membrane. The contact-dependence is understandable in light of the data indicating a requirement for CD40/CD40L in order to produce full activation of tryptophan degradation.

Example 2: Tryptophan degradation by MCSF-derived Macrophages

- 25 *Medium in macrophage-lymphocyte co-cultures is selectively depleted of tryptophan.* Conditioned medium was generated from co-cultures of macrophages plus T cells activated with mitogen (two different mitogens, anti-CD3 antibody and SEB, gave equivalent results; the data presented are from anti-CD3). Fresh lymphocytes were suspended in
- 30 conditioned medium and activated with additional mitogen. Care was taken to exclude all traces of fresh medium.

- As shown in Figure 1, conditioned medium completely failed to support T cell proliferation (less than 1% of the proliferation in fresh medium). In contrast, control conditioned media from macrophages alone,
- 35 from co-cultures of macrophages and T cells without mitogen, and from T cells activated with fresh monocytes instead of macrophages, all supported T

5 cell proliferation comparably to fresh medium (90-140%) of control, n=3-4
in each group, p=NS by ANOVA, see Munn J. Immunol. 1996;156:523-532
for representative data.

Selective add-back studies showed that the deficiency lay in an
essential amino acid. Supplementation with individual amino acids (Figure
10 1) showed that tryptophan was depleted in conditioned medium. The
addition of tryptophan to conditioned medium fully restored T cell
proliferation, indicating that tryptophan was the only deficient component.
Consistent with this, HPLC analysis of conditioned media showed that the
levels of other essential amino acids were similar to fresh medium (some
15 concentrations were higher, presumably due to protein catabolism).

As shown in Figure 2, titration of reagent tryptophan in conditioned
medium gave a half-maximal concentration of approximately 500 nM for T
cell proliferation (compared to a normal level in RPMI of 25 μ M). These
studies also showed that as little as 50 nM was sufficient to initiate low but
20 readily detectable T cell proliferation, indicating that tryptophan had been
reduced below this range. Consistent with this, direct measurement of
tryptophan in conditioned medium was below the detection limit for our
assay, which was 50 nM.

Depletion of tryptophan is rapid. Macrophages were co-cultured
25 with T cells and mitogen for 24 hrs to allow up-regulation of the tryptophan-
depletion pathway, then fresh medium was added and the kinetics of
elimination measured. As shown in Figure 3, tryptophan was eliminated by
first-order kinetics with a half-life of approximately 2 hours. The initial rate
of elimination (when tryptophan was not limiting) was up to 20,000
30 pmol/ 10^6 cells/hr. By way of comparison, fresh monocytes stimulated with
IFN γ (a standard model of IDO induction) degraded tryptophan at
approximately 200 pmol/ 10^6 cells/hr, which is consistent with the literature.
This rapid rate of tryptophan depletion could not be attributed to
consumption by cellular metabolism. Despite their high metabolic activity,
35 macrophages alone depleted tryptophan at a barely detectable rate (less than
5% of the activated rate, Figure 3). It also did not reflect sequestration of

5 free tryptophan within macrophages, since the preliminary validation studies had shown that tryptophan was undetectable in culture supernatants whether macrophages were intact or lysed prior to performing the assay.

Example 3: Inhibition of Tryptophan degradation by MCSF-derived Macrophages prevents Suppression of T cells.

10 *Inhibition of tryptophan degradation prevents Macrophage-mediated suppression of T cells.* The data predicts that pharmacologic inhibitors of IDO should prevent suppression. One potent inhibitor of IDO is the 1-methyl derivative of tryptophan (Cady, et al. Arch. Biochem. Biophys. 1991;291:326-333). As shown in Figure 4A, the presence of 1-methyl-tryptophan effectively prevented macrophages from suppressing T cell
15 activation. These data were selected in order to clearly demonstrate that suppression can be abrogated, but in most experiments the effect of 1-methyl-tryptophan has been less complete (typically 59-60% reversal of suppression). This was not unexpected, given the extremely high level of
20 enzyme activity and the fact that 1-methyl-tryptophan is a competitive, rather than an irreversible, inhibitor. In order to fully abrogate macrophage-mediated suppression it will be necessary to simultaneously block the induction of the enzyme and to pharmacologically inhibit its activity.

Macrophages can overcome high concentrations of tryptophan. A
25 second test of the mechanism was to supplement the culture medium with additional tryptophan to reverse the inhibitory effect of IDO. Tryptophan supplementation alone was never as effective as 1-methyl-tryptophan, and sometimes was entirely ineffective, and as shown in Figure 4B, appears to be related to the seeding density of macrophages and T cells. At low densities,
30 supplemental tryptophan effectively reversed suppression, but at high densities the macrophages remained able to suppress. The effect of seeding density is believed to reflect the limited ability of diffusion to deliver tryptophan to the T cells and macrophages.

Example 4: Effect of Cytokines on Macrophage Tryptophan Degradation.

35 *Macrophage tryptophan degradation is synergistically induced by*

5 *signals of early T cell activation.* The rapid depletion of tryptophan by
macrophages was not induced by resting T cells, but occurred only when the
T cells attempted to activate (cf. Figure 3). This implied that the
macrophages were detecting some sign of T cell activation which triggered
tryptophan degradation. In light of the studies implicating IFN γ as a major
10 regulator of the IDO gene, it was predicted that T cell-derived IFN γ would be
the signal for tryptophan degradation. However, when dose-response
titration was performed of recombinant IFN γ on MCSF-derived
macrophages, it was found that IFN γ concentrations of 100 U/ml or more
were required for maximal induction, and even then the level of enzyme
15 activity did not reach those achieved by co-culture with activated T cells.

CD40L is upregulated early in T cell activation and it is known to act
synergistically with IFN γ in signaling macrophages for other functions. The
response of MCSF-derived macrophages to IFN γ in the presence or absence
of recombinant CD40L (prepared as a homo-trimer in order to signal via
20 CD40 in soluble form, gift of Bill Fanslow, Immunex Corporation) was
compared. The addition of CD40L alone had little effect on enzyme activity,
but as shown in Figure 5 it displayed marked synergy when combined with
IFN γ . The effect of CD40L was to shift the dose-response curve of IFN γ one
to two orders of magnitude, so that significant response now occurred at
25 IFN γ concentrations as low as 1 U/ml. Thus, two signals normally delivered
by T cells to APC early in the course of activation combined to induce
tryptophan degradation in MCSF-derived M ϕ s.

*The responsiveness of tryptophan metabolism to IFN γ and CD40L is
developmentally regulated.* It was then determined if all monocyte-derived
30 cells degraded tryptophan in response to these signals, or whether it was
specifically associated with the MCSF phenotype. To generate a non-
suppressive phenotype for comparison, monocytes were cultured for 5 days
GMCSF+IL-4, which produces cells that closely resemble tissue dendritic
cells, and which are potent activators of T cells. For clarity, these cells are
35 referred to as dendritic cells and MCSF-derived cells as macrophages. As
shown in Figure 6, dendritic cells showed minimal tryptophan degradation in

5 response to IFN γ and CD40L, in contrast to MCSF-derived macrophages. Thus, the response to IFN γ was not simply a feature of differentiated monocytes, but was regulated by the cytokine milieu present during terminal differentiation. Interestingly, when the cytokines were added sequentially, the effect of CD40L on dendritic cells was to further inhibit any residual
10 induction of tryptophan degradation in response to IFN γ , exactly the opposite of its effect on macrophages.

Example 5: The tryptophan transport system in Macrophages

Transport of tryptophan into Macrophages is developmentally regulated. Whether the upregulation of tryptophan degradation was
15 accompanied by increased or decreased tryptophan transport into macrophages was then determined. As shown in Figure 7A, following differentiation MCSF-derived macrophages showed a markedly increased ability to take up tryptophan, 10 to 20-fold greater than fresh monocytes, which was further induced by activation with IFN γ .

20 *Tryptophan transport in MCSF-derived Macrophages shows a preferential affinity for tryptophan.* To characterize the tryptophan transport system, whether uptake was dependent on the sodium gradient was then determined. Preliminary studies had shown that tryptophan transport in fresh monocytes was sodium dependent (less than 10% of transport was sodium-
25 independent). However, as shown in Figure 7B, the majority of the tryptophan transport in macrophages was independent of sodium. This sodium-independent system was then characterized using cross-competition studies. As expected, T cells showed a pattern consistent with system L, with tryptophan uptake being inhibited by aromatic and neutral amino acids,
30 but not lysine or arginine (Figure 8A). In contrast, however, tryptophan uptake by MCSF macrophages (Figure 8B) was only partially inhibited by neutral amino acids, and even phenylalanine could not fully block it.

As shown in Figure 9, titration studies indicated that phenylalanine (the best competitor) was 10- to 100-fold less effective than tryptophan in
35 competing for uptake, based on relative IC₅₀. Tyrosine, leucine, and the system L-specific substrate 2-amino-2-norbornane-carboxylic acid (BCH)

5 were even less effective competitors. Significantly, when system L was inhibited by 8 mM BCH, there still remained 30-50% of the total tryptophan transport available (depending on the experiment). Taken together these data suggested that a high-affinity, non-system L transport pathway might be present.

10 **Example 6: In vivo studies of the role of IDO and tryptophan degradation on activation of T cells.**

Peripheral deletion of autoreactive T cells in vivo is accompanied by evidence of tryptophan degradation. Whether there was evidence that IDO was involved in peripheral tolerance *in vivo* was then assessed. Since IDO
15 activity at the single cell level cannot be measured *in vivo*, a model in which a large number of autoreactive T cells underwent synchronous activation and deletion was needed. This would allow one to follow the kynurinine breakdown products released systemically when IDO degrades tryptophan (Meyer, et al. *J. Lab. Clin. Med.* 1995;126:530-540). Dr. Mellor had
20 developed such a model by creating mice transgenic for a T cell receptor recognizing the H-2K^b (MHC class I) antigen. When these T cells are adoptively transferred into a syngeneic animal made transgenic for the target antigen, they become functionally autoreactive. As described in Tarazona, et al. *Intl. Immunol.* 1996;8:351-358, these autoreactive T cells undergo a
25 transient period of activation, but then are rapidly eliminated (day 4-5) without causing disease. This occurs despite the fact that they are not subject to rejection by the host (being syngeneic), and are eliminated only in animals where they encounter their antigen. This therefore represents a useful model of synchronous deletion of autoreactive T cells.

30 Splenocytes from T cell receptor-transgenic mice (4×10^7 cells) were adoptively transferred by tail vein injection into antigen-transgenic mice. On day 3 (at the beginning of the rapid expansion phase), the recipient animals were sacrificed and serum was obtained. Pre-transfer sera were used as controls. As shown in Figure 10, mice which had received autoreactive T
35 cells showed a marked elevation in serum kynurinine compared to controls. Thus, the expansion and elimination of autoreactive T cells *in vivo* was

5 accompanied by evidence of significant tryptophan catabolism, consistent with activation of IDO.

Whether *in vivo* inhibition of the IDO enzyme with 1-methyl tryptophan (as described *in vitro* in Figure 4A) would alter the kinetics of activation or elimination of autoreactive T cells in the mouse model was then
10 measured. As shown in Figure 11, continuous infusion of 1-methyl tryptophan (via subcutaneously implanted timed-release pellets, 1000 mg/kg/day) resulted in significantly enhanced activation of the adoptively transferred T cells. These results strongly support a role for IDO in suppressing T cell activation *in vivo*.

15 **Example 7: MCSF and tissue Macrophages.**

Clinically, there exists an ill-defined association between altered tryptophan metabolism and autoimmune disorders. Patients receiving L-5-hydroxytryptophan for neurologic disorders experienced a high frequency of a scleroderma-like illness (Sternberg *N. Engl. J. Med.* 1980;303:782-787).
20 Individuals who ingested certain preparations of L-tryptophan contaminated with toxic tryptophan derivatives (1-1' ethylenedibis-[tryptophan]) experienced the eosinophilia-myalgia syndrome (Belongia *N.E. Engl. J. Med.* 1990;323:357-365; Mayeno *Science* 1990;250:1707-1708), which included excessive T cell activation. There is even a case report of simple dietary
25 supplementation with tryptophan leading to multiple autoimmune disorders (Morgan, et al. *Br. J. Dermatol.* 1993;128:581-583).

The osteopetrotic (*op/op*) mouse lacks functional MCSF, and has a selective deficiency in specific subsets of tissue macrophages (Ceccini, et al. Development 1994;120:1357-1372). It has normal dendritic cell
30 development and normal T cell responses to foreign antigens (Begg *J. Exp. Med.* 1993;177:237-242) indicating that these functions are not MCSF-dependent. Interestingly, in one well-established model of spontaneous autoimmunity, the NOD mouse, the macrophages have been shown to have a defective proliferative response to MCSF, and fail to undergo MCSF-
35 induced terminal differentiation (Serreze, et al. *J. Immunol.* 1993;150:2534-2543). This led to speculate that autoimmune NOD mice lack a specific,

5 MCSF-induced tolerogenic macrophage subset. The *in vitro* studies show that MCSF is a growth factor for both immunosuppressive and inflammatory macrophages, and that when inflammatory cytokines are present they act dominantly over MCSF. Thus, in the presence of dysregulated inflammatory cytokines, the addition of MCSF would be expected to exacerbate rather than
10 reduce the disorder (Moore, et al. J. Immunol. 1996;157:433-440).

Based on the preliminary data, it was proposed that IFN γ induces IDO in certain macrophage and thereby triggers T cell suppression. This is a somewhat surprising role for IFN γ , which is generally considered pro-inflammatory rather than immunoregulatory. However, mice with targeted
15 disruptions of the gene for either IFN γ (Krakowsk, et al. Eur. J. Immunol. 1996;26:1641-1646) or the IFN γ receptor (Willenborg, et al. J. Immunol. 1996;157:3223-3227) demonstrate an unexpected hyper-susceptibility to experimental autoimmune encephalomyelitis (EAE). Mice normally resistant to this induced autoimmune disorder are rendered susceptible in the
20 absence of IFN γ , and the disease is fatal and uncontrolled. Adoptive transfer studies showed that the requirement for IFN γ lay with an IFN γ -responsive regulatory cell, not with the autoreactive T cells. In contrast, these mice fail to mount an appropriate inflammatory response to intracellular pathogens (Dalton, et al. Science 1993;259:1739-1742; Kamijo, et al. J. Exp. Med
25 1993;178:1435-1440). Thus, IFN γ is pro-inflammatory in infectious disease, but is immunoregulatory in at least one model of autoimmunity. This is consistent with the hypothesis that some macrophages respond to IFN γ as a signal to inhibit T cell activation.

The following examples demonstrate that 1-methyl-L-tryptophan, an
30 inhibitor of L-tryptophan degradation, administered during murine pregnancy induces loss of allogeneic (mother and fetus are genetically different) conceptus 2-3 days after blastocyst implantation in uterus. Syngeneic conceptus are not affected by this treatment.

**Example 8: The mIDO gene is transcribed in epididymis and in
35 conceptus during pregnancy.**

RT-PCR analyses on RNA extracted from a panel of mouse tissues

5 was conducted to examine the extent of mIDO gene transcription in mice. mIDO transcripts were detected in RNA samples from mouse epididymis but not from muscle, heart, bone-marrow, spleen, peripheral lymph nodes, kidney, liver, brain, intestine or lung. PCR products were generated by RT-PCR amplification from RNA samples prepared from pooled syngeneic (s) or allogeneic (a) conceptus. RT-PCR amplification of murine α -actin transcripts was conducted on each RNA sample to verify RNA integrity.

10 Methods: Female CBA mice were mated with syngeneic or allogeneic (B6) male mice. Females were inspected daily (am) for vaginal plugs; the morning plugs were detected was taken as 0.5 dpc (days *post coitus*). All conceptus dissected from each pregnant female were pooled, snap frozen on liquid nitrogen and used or prepare total RNA using standard procedures by homogenization in RNA-STAT 60 solution (Tel-TestB Inc.). Transcripts of the murine IDO gene (Habara-Ohkubo, et al., 1991 Gene 105) were detected by RT-PCR using forward (GTACATCACCATGGCGTATG) and reverse

15 (GCTTTCGTCAAGTCTTCATTG) oligonucleotide primers which generated PCR products of the expected size (740bp. RT-PCR conditions used were 48°C, 45 min./94°C, 2 min. (1 cycle); 94°C, 30 sec./58°C, 1 min./68°C, 2 min. (40 cycles); 68°C 5 min. (1 cycle). PCR products were fractionated on a 1.5% agarose/TBE gel containing ethidium bromide and

20 were visualized by UV fluorescence and images of gels were recorded as a digital bitmaps using a high definition digital fluorescence and images of gels were recorded as a digital bitmaps using a high definition digital camera. RT-PCR amplification of the murine α -actin gene (480bp) was performed in parallel to verify RNA integrity.

25 In all cases, PCR products from the α -actin gene were detected indicating that RNA samples were not degraded.

IDO gene expression has been described in human placental trophoblast cells. To examine whether IDO transcription occurs during murine pregnancy, RNA samples were prepared from uterus and conceptus

30 of pregnant mice at various stages of gestation. Female mice (CBA) were mated to syngeneic (CBA) or allogeneic C57BL/6 (B6) male mice and

5 inspected daily for vaginal plugs. The morning the plug was detected was taken as 0.5 days post coitus (dpc). RNA samples were prepared from dissected components of conceptus; embryos, decidua (extra-embryonic trophoblast plus maternal uterus) and from uterus tissues. mIDO gene transcripts were detected in conceptus, but not uterus from syngeneic and
10 allogeneic conceptus at early (7.5dpc, 9.5dpc) gestation times. mIDO transcripts were not detected in embryo RNA at 9.5dpc suggesting that decidual tissues were the site of IDO expression in the conceptus. At later gestation times (10.5 and 13.5dpc) mIDO transcripts were detected in allogeneic conceptus but not in syngeneic conceptus. RNA was prepared
15 from decidual tissues dissected separately from embryos. mIDO transcripts were not detected in RNA from embryos at 10.5 or 13.5dpc. From these data it was concluded that mIDO transcription occurs in decidual tissues of all conceptus from early gestation times (7.5-9.5dpc).

**Example 9: Loss of allogeneic conceptus occurs in the presence of an
20 inhibitor of IDO enzyme activity.**

A study was conducted to test whether IDO enzyme activity contributes to survival of fetal allografts by treating pregnant female mice with 1-methyl-L-tryptophan, an inhibitor of IDO enzyme function. Female (CBA) mice were mated with syngeneic (CBA) or allogeneic (B6) male
25 mice. On 4.5 days post coitus ("dpc") two slow-release pellets impregnated with 1-methyl-L-tryptophan (1g/kg/day) were surgically implanted under the dorsal skin of pregnant mice. Control mice were treated with pellets not impregnated with 1-methyl-L-tryptophan (placebo groups). After surgery, mice were sacrificed and their uterus examined macroscopically and
30 microscopically at various stages of gestation.

Based on comparison with extensive breeding records from the MCG Transgenic Unit fecundity rates for mouse colonies bred at MCG are 6.8 (CBA x CBA) and 6.4 (CBA x B6) pups per female at parturition. The same procedure was used to assess embryo survival and development in the mating
35 combinations presented in Table 2. A large cohort of untreated female CBA mice mated with GK transgenic male mice were examined and are included

- 5 in Table 1 to show that the mean number of conceptus (at parturition) is identical to the [CBA x B6] mating combination.

Results are summarized in Table 1.

Table 1 - Inhibition of IDO activity induces rejection of allogeneic conceptus					
Mating Genotype conceptus	Gestation Stage	Mean No. conceptus/♀; (No. ♀ treated)		Appearance of	
♀ x ♂	(dpc)	Inhibitor	Placebo	Inhibitor	Placebo
CBA x CBA	6.5-8.5	7.3(3)	7.5(2)	normal	normal
	15.5	6.7(6)	6.5(6)	normal	normal
CBA x B6	6.5	7.3(3)	8.5(2)	normal	normal
	7.5	4.5* (6)	8.0 (4)	majority inflamed	normal
	8.5/9.5	0.5* (17)	7.3 (6)	all inflamed	normal
	11.5-15.5	0* (7)	5.8 (4)	-	normal
*-p<0.05 by ANOVA compared to placebo controls at each time point					

- 10 Treatment with 1-methyl-L-tryptophan had a profound affect on development of allogeneic conceptus. Syngeneic and allogeneic conceptus in mice treated with 1-methyl-L-tryptophan were found in normal numbers, were all healthy and were at the appropriate stage of fetal development at 6.5dpc. Examinations conducted at 7.5dpc, however, revealed striking
- 15 differences in the numbers and appearances of conceptus. The average number of allogeneic conceptus + inhibitor was reduced to 4.5 per female at 7.5dpc and declined to <2 at 8.5/9.5dpc. No conceptus were present on any mice carrying allogeneic conceptus and treated with 1IDO inhibitor after 9.5dpc. Normal numbers of healthy allogeneic (-IDO inhibitor) and
- 20 syngeneic (±IDO inhibitor) conceptus were present in control groups.
- Methods. Mice were mated, treated with IDO inhibitor and dissected at gestation times indicated. Tissues were prepared for sectioning by fixing them in 4% paraformaldehyde. Serial sections (5µm) were prepared using a

5 microtome and were stained with hemtoxylin and eosin before microscopic examination.

On gross examination significant inflammation and excessive maternal blood surrounded the majority of 7.5dpc allogeneic conceptus in mice treated with IDO inhibitor. In contrast, maternal blood was localized
10 normally near the ectoplacental cone in all 7.5dpc syngeneic conceptus treated with IDO inhibitor; their appearance was indistinguishable from that if syngeneic and allogeneic conceptus dissected from mothers in placebo groups.

On histological examination increased numbers of enlarged blood
15 vessels within the decidual region were observed in 7.5dpc allogeneic conceptus when IDO activity was blocked. However, allogeneic 7.5dpc embryos appeared intact and at the expected developmental stage. One day later (8.5 dpc) allogeneic conceptus (+IDO inhibitor) were grossly abnormal. Extensive mixed mononuclear and neutrophil infiltrates were evident in
20 every conceptus and were accompanied by extensive tissue degeneration in the decidual region. Grossly abnormal embryos were present but developmentally retarded. At 9.5 dpc no allogeneic embryos were present in mice treated with IDO inhibitor and decidual swellings were not apparent; some cellular debris was detected within the uterine lumen.

25 These data reveal that blocking IDO activity during pregnancy has a profound and specific effect on development of allogeneic conceptus, leading to loss of all embryos by mid-gestation. Embryo loss was not caused by surgical manipulations to implant pellets at 4.5 dpc. This data shows that blocking IDO activity compromises development of conceptus only when
30 there is a genetic difference between mother and embryos. This implies that functional IDO enzyme activity is essential to protect allogeneic conceptus during early gestation and that the maternal immune system is involved in processes that lead to loss of allogeneic conceptus.

5 **Example 10: Maternal lymphocytes provoke loss of allogeneic conceptus when IDO activity is blocked.**

The contribution of maternal lymphocytes to experimentally induced loss of allogeneic conceptus in mice treated with 1-methyl-L-tryptophan was assessed using females carrying the RAG-1 induced mutation (RAG-1^{-/-}) which prevents development of T or B cells (Li, 1982, Cancer 50, 2066-2073). As before, female mice carrying disrupted RAG-1 genes (on the DBA background) were mated with B6 males and treated with 1-methyl-L-tryptophan (4.5cpd). Pregnancies were continued until 11.5dpc when females were sacrificed to assess the number of conceptus. Normal numbers of healthy conceptus were found in all females examined (Table 2, first line).

Table 2 - Maternal lymphocytes and a single MHC I alloantigen induce embryo rejection

Mating Genotype No.	IDO	No. ♀ examined	Mean
(♀ x ♂)	Inhibitor	(dpc)	conceptus/♀
CBA [RAG-1 ^{-/-}] x B6	yes	5 (11.5)	7.8
CBA [RAG-1 ^{-/-}] x B6	yes	2 (20.5, post partum)	7 (pups born)
CBA x GK*	yes	4 (11.5)	0
CBA x GK*	no	21 (11.5)	6.4

*GK mice are H-2K^b-transgenic mice made on the CBA background.

This data demonstrates that maternal lymphocytes are essential participants in events that precede loss of allogeneic conceptus when IDO activity is blocked. The most likely explanation is that maternal T cells are responsible for initiating mediating processes that lead to loss of allogeneic conceptus when IDO activity is blocked. This premise is based on the assumption that conceptus, like tissue allografts, provoke T cell responses. Although B cells, via antibody production, can contribute to allograft rejection, it is unlikely that antibodies against fetal alloantigens mediate rapid loss of conceptus a few days after implantation, especially as virgin females were used in these experiments.

5 **Example 11: A single MHC I difference provokes loss of allogeneic conceptus.**

B6 and CBA mice differ at a large number of genetic loci including the entire MHC region (MHC I and MHC II) as well as at multiple minor histocompatibility (miHC) loci. To determine whether a single polymorphic
10 MHC molecule could provoke fetal loss, CBA mice were mated with (GK) transgenic male mice carrying a recombinant H-2K^b (GK) transgene; a promoter from the human HLA-G gene drives H-2K^b-expression at high level in murine trophoblast (Zhou, et al. 1998 *J. Reprod. Immunol.* 40, 47-62). GK mice were made on the inbred CBA strain background and, hence,
15 differ from CBA mice only because of H-2K^b expressed by the GK transgene. Normal size litters are borne by CBA females after mating with GK transgenic male mice (Table 2). Pregnant mice were treated with 1-methyl-L-tryptophan and examined at 11.5dpc, as before, to assess effects on fetal development (Table 2). No conceptus, nor any resorbed decidua, were
20 observed when mice were examined on 11.5dpc, although, in each mouse, the uterus was distended indicating that pregnancy had terminated prematurely.

This result is important for two reasons. First, a single paternally-inherited MHC I difference between mother and conceptus is enough to
25 provoke fetal loss when IDO activity is blocked. Second, it eliminates the possibility that fetal loss occurs after CBA [x B6] matings because of an inherent genetic predisposition to fetal loss in this mating combination.

This result supports the view that maternal T cells, not B cells, are responsible for fetal loss since MHC alloantigens provoke strong T cell
30 responses but weak B cell responses. If true, this implies that maternal CD8⁺ T cells recognizing native H-2K^b molecules expressed by fetal cells may be responsible for provoking fetal loss.

In summary, these data demonstrate that the IDO enzyme inhibitor 1-methyl-tryptophan has a profound and specific abortifacient effect on all
35 allogeneic conceptus prior to 9.5dpc. Fetal abortion is not due to non-specific toxic affects of the drug itself as syngeneic conceptus develop

5 normally in females exposed to 1-methyl-tryptophan as do allogeneic
conceptus in the absence of maternal lymphocytes. Alternatively, embryonic
development may be affected by substances produced when 1-methyl-
tryptophan is metabolized by cells expressing IDO. However, this is
unlikely to explain the abortifacient effect of 1-methyl-tryptophan since the
10 IDO gene is expressed in syngeneic and allogeneic conceptus early in
gestation during the period when embryo rejection occurs (7.5-9.5dpc). One
therefore concludes that the abortifacient effect of 1-methyl-tryptophan
arises because it inhibits IDO enzyme activity. Decidual cells expressing
IDO normally establish a protective barrier that prevents maternal T cell
15 mediated rejection of allogeneic conceptus.

**Example 12. Inhibition of Tumor Growth by administration of IDO
inhibitor.**

In light of the data herein showing that the allogeneic fetus is able to
protect itself against attack by the maternal immune system through
20 expression of IDO, and given the expression of IDO in tumors as cited
above, it was tested whether a similar mechanism allowed tumor cells to
evade immune attack. The MB49 epithelial carcinoma model was chosen for
proof of concept because it is an aggressive, lethal malignancy when
innoculated into syngeneic hosts, but it can be rendered susceptible to
25 immune attack if the host's immune system is suitably activated (Chen, et al.
1997 *J. Immunol.* 159, 351-359). Tumor-bearing hosts were treated with the
IDO inhibitor 1-methyl-tryptophan. MB49 tumor cells (1×10^6) were
injected subcutaneously into syngeneic C57/Bl6 hosts. Pellets containing 1-
methyl-tryptophan (0.9 mg/hr, 7-day release) were implanted at the time of
30 tumor cell inoculation. By day 10, all animals had evidence of initial tumor
formation (palpable mass). By day 15, control animals were visibly ill and
the experiment was terminated. Animals were sacrificed on day 11-15 for
histologic examination.

As shown in Table 3, administration of 1-methyl-tryptophan
35 significantly reduced tumor growth in immunocompetent, syngeneic hosts,
compared to vehicle control. Two important points are noted: First, no

- 5 manipulation of the immune system other than inhibition of IDO was required for anti-tumor effect – i.e., the tumor itself became immunogenic once the barrier of IDO was removed. Second, the observed responses continued at least 4-7 days *after* the period of inhibitor administration (days 1-7), suggesting that an initial priming period was sufficient to generate a
- 10 sustained anti-tumor immune response.

Table 3. Anti-tumor effects of 1-methyl-tryptophan administration.

Outcome measure	control group (n=7)	1-methyl-tryptophan group (n=7)
complete responses	0	1
partial responses	0	6
size of residual tumor	2 - 2.5 cm	1 cm (necrotic)
histology (day 15)	viable tumor, minor infiltration of CD4+ T cells with occasional CD8+, scattered macrophages	apoptosis, necrosis, and hemorrhage, extensive infiltrate of CD4+ and CD8+ T cells, extensive infiltration of macrophages
visible metastases	extensive local metastases, with gross evidence of intraperitoneal and visceral metastases	none
performance status	decreased activity, failure to groom, ill appearance	normal

- 15 Modifications and variations of the methods and compositions described herein will be obvious to those skilled in the art and are intended to be encompassed by the claims.

We claim:

1. A method for altering T cell mediated immunity comprising altering local extracellular concentrations of tryptophan and tryptophan metabolites.
2. The method of claim 1 wherein the extracellular concentration is altered by inhibition or increased activity of IDO mediated metabolism of tryptophan.
3. The method of claim 1 wherein the tryptophan concentrations are decreased below physiological levels.
4. The method of claim 1 wherein the tryptophan concentrations are increased above physiological levels.
5. The method of claim 1 wherein the concentrations are altered to regulate rejection or acceptance of a allograft or xenograft
6. The method of claim 5 wherein the concentrations of tryptophan are decreased or the concentration of tryptophan metabolites are increased to prevent or terminate pregnancy.
7. The method of claim 5 wherein the concentrations of tryptophan are increased or the concentration of tryptophan metabolites are decreased to promote pregnancy.
8. The method of claim 5 wherein the concentrations of tryptophan are increased to decrease the likelihood of rejection of an allograft or xenotransplanted tissue or organ.
9. The method of claim 1 wherein the concentrations of tryptophan or tryptophan metabolites are altered to induce rejection of cells by a human or animal hosting the cells.
10. The method of claim 9 wherein the cells are tumor cells.
11. The method of claim 9 wherein the cells are virally infected or transformed cells.
12. The method of claim 1 wherein compounds decreasing tryptophan degradation are administered in combination with a vaccine to enhance the immune reaction to the vaccine.
13. The method of claim 1 wherein compounds decreasing tryptophan degradation are administered to help reverse the immunosuppressed state

found in AIDS

14. The method of claim 1 wherein compounds increasing tryptophan degradation are administered to decrease an autoimmune response.
15. The method of claim 1 wherein the concentration of tryptophan is altered by administration of a compound inhibiting tryptophan degradation.
16. The method of claim 1 wherein the concentration of tryptophan is altered by administration of a compound increasing extracellular levels of tryptophan.
17. The method of claim 1 wherein the concentration of tryptophan is altered locally or topically.
18. The method of claim 1 wherein the concentration of tryptophan is altered systemically.
19. The method of claim 1 wherein tryptophan concentration is decreased further comprising administering a cytokine to increase tryptophan degradation.
20. The method of claim 19 wherein the cytokine is MCSF.
21. The method of claim 19 wherein the composition and cytokine is administered to a patient with an autoimmune disorder.
22. The method of claim 19 wherein the composition and cytokine is administered to a patient with an allograft or xenograft.
23. A method for assessing the immunological state of certain cells comprising measuring tryptophan levels, tryptophan metabolite levels or IDO activity.
24. The method of claim 23 wherein the cells are a tissue biopsy from a patient suspected of having a malignancy.
25. The method of claim 23 wherein the cells are obtained from a pregnant woman.
26. A pharmaceutical composition for altering extracellular tryptophan concentrations comprising
an effective amount of a compound suitable for administration to a patient in need of treatment thereof to alter extracellular levels of tryptophan or tryptophan metabolites to modify T cell mediated immunity, and

- a pharmaceutically acceptable carrier for administration to a patient.
27. The composition of claim 26 increasing tryptophan concentrations and decreasing tryptophan metabolite concentrations in extracellular fluid.
 28. The composition of claim 26 decreasing tryptophan concentrations and increasing tryptophan metabolite concentrations in extracellular fluid.
 29. The composition of claim 27 comprising an inhibitor of IDO.
 30. The composition of claim 29 wherein the inhibitor is selected from the group consisting of 1-methyl-DL-tryptophan, β -(3-benzofuranyl)-DL-alanine, β -[3-benzo(b)thienyl]-DL-alanine, and 6-nitro-L-tryptophan).
 31. The composition of claim 28 comprising an enzyme degrading tryptophan.
 32. The composition of claim 31 selected from the group consisting of recombinant IDO and indolyl-3-alkane alpha-hydroxylase.
 34. The composition of claim 26 comprising an inhibitor of the high affinity, sodium independent tryptophan transport system.
 35. The composition of claim 26 in a formulation for controlled or sustained release.
 36. The composition of claim 26 further comprising a vaccine.
 37. The composition of claim 26 in a dosage and formulation for injection into synovial joints to decrease an autoimmune response.
 38. The composition of claim 26 in a dosage and formulation for intravaginal administration for preventing or terminating pregnancy.

1/6

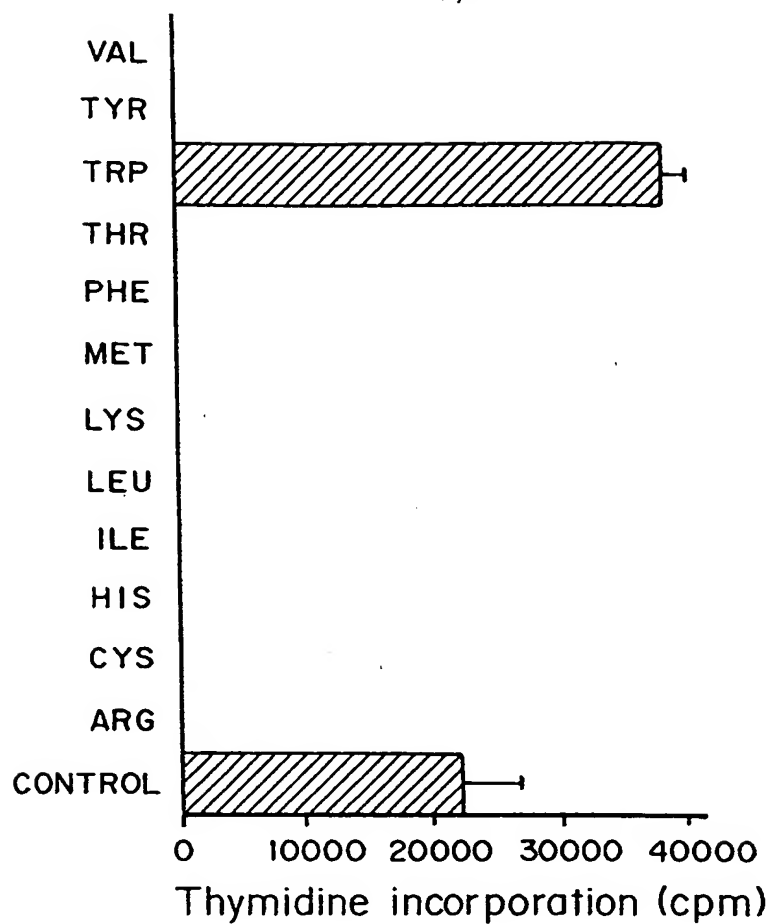


FIG. 1

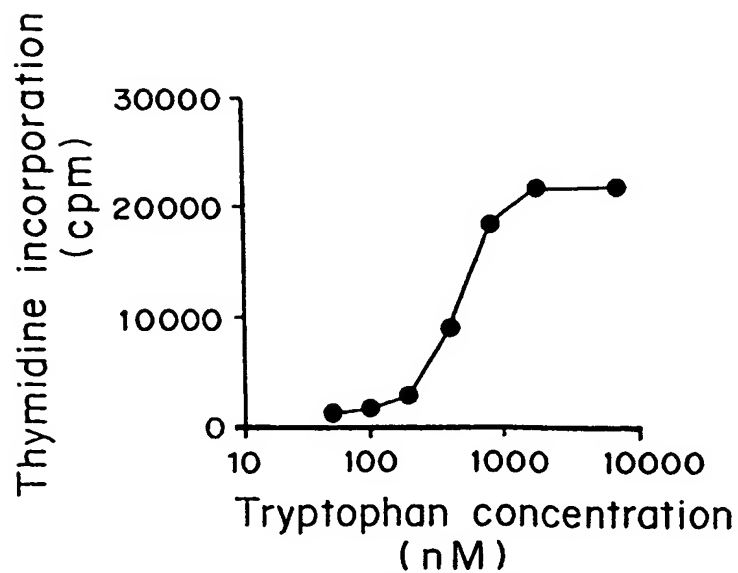


FIG. 2

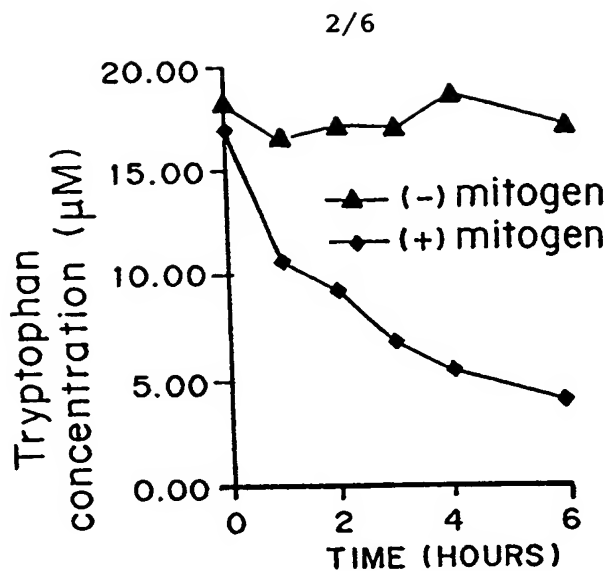


FIG. 3

FIG. 4A

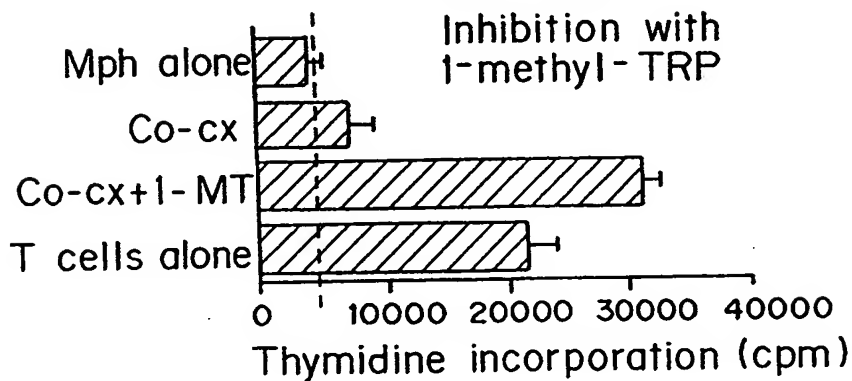
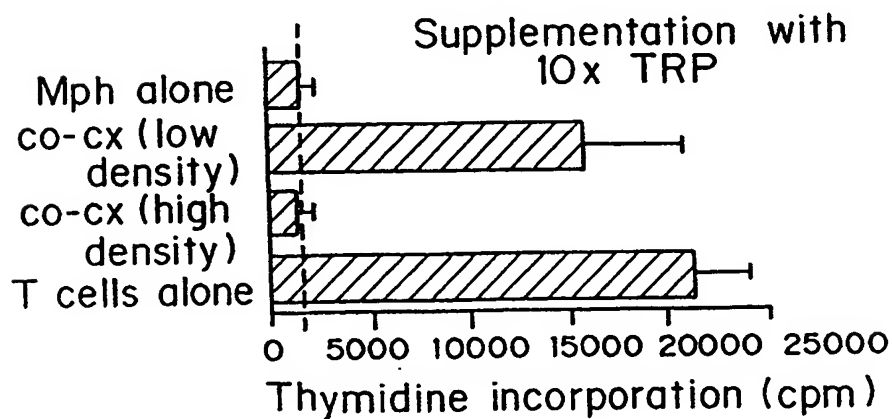


FIG. 4B



3/6

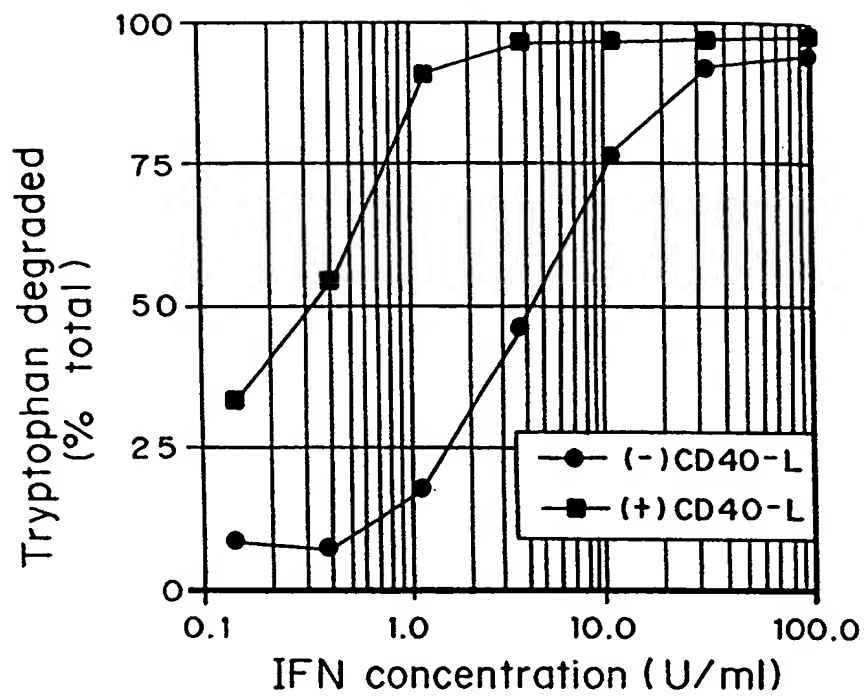


FIG. 5

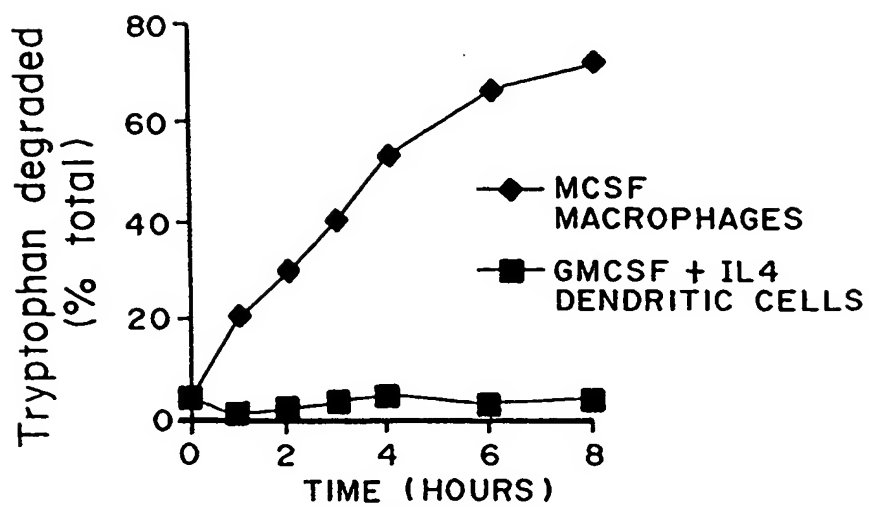


FIG. 6

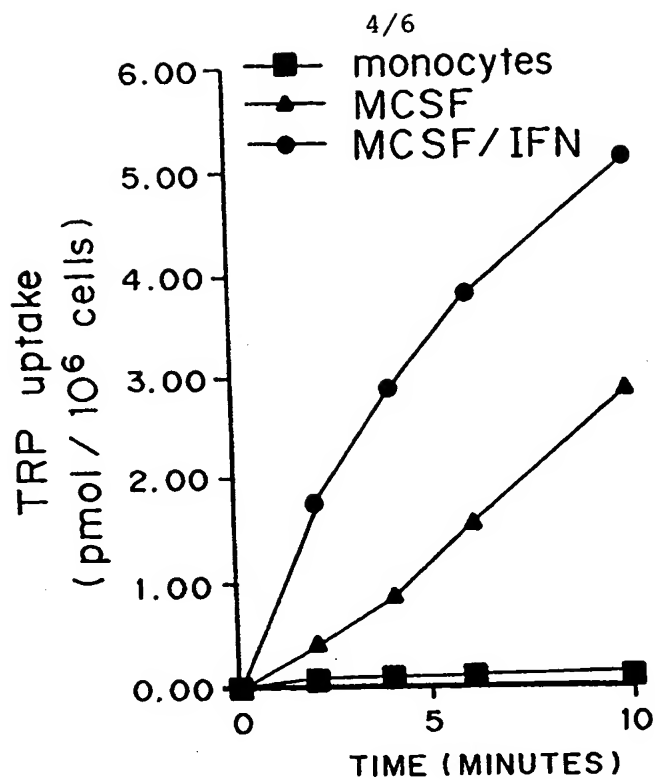


FIG. 7A

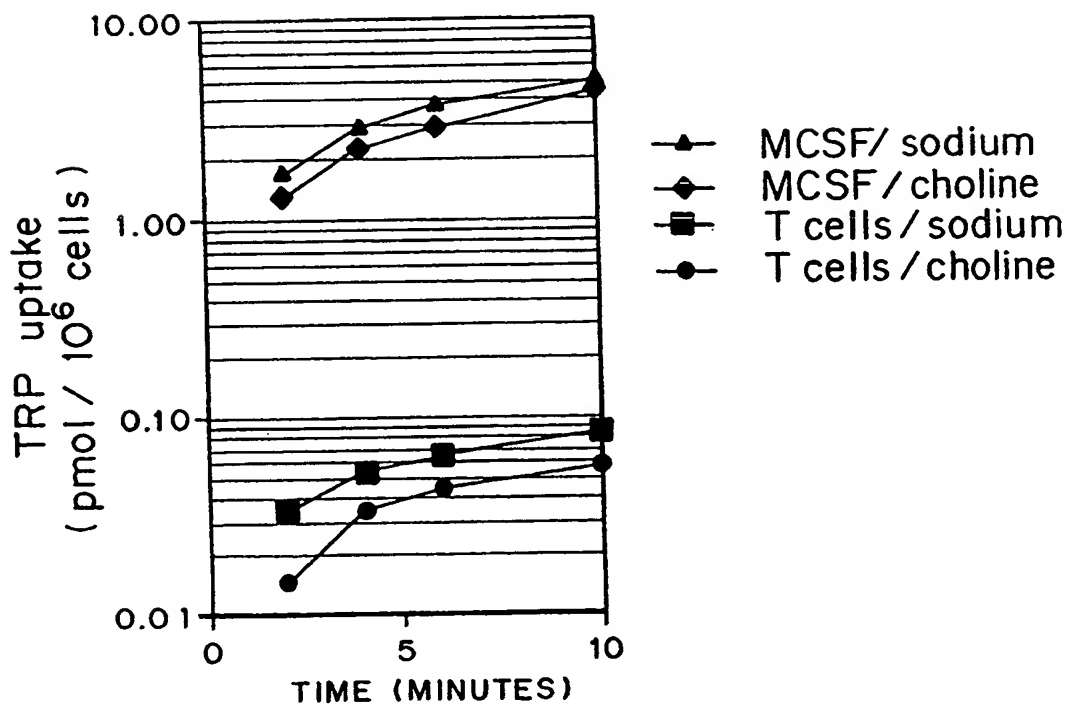


FIG. 7B

5/6

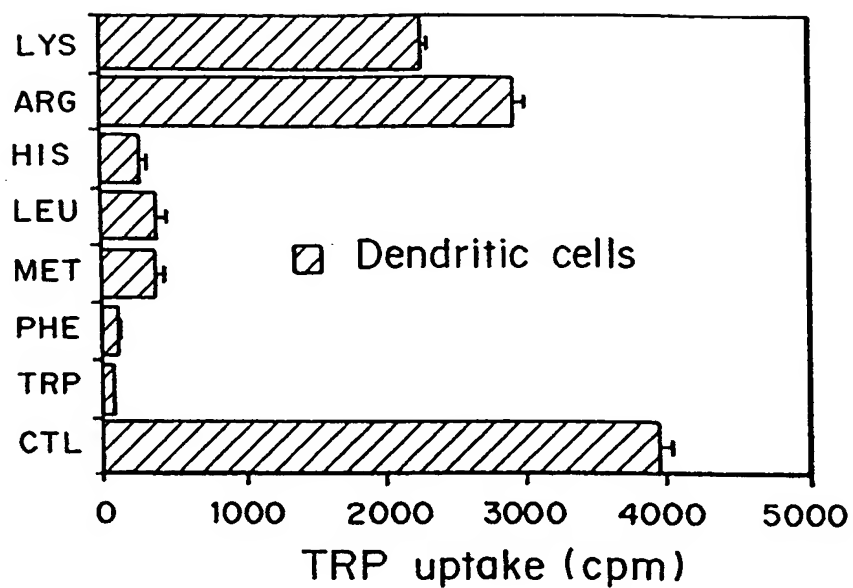


FIG. 8A

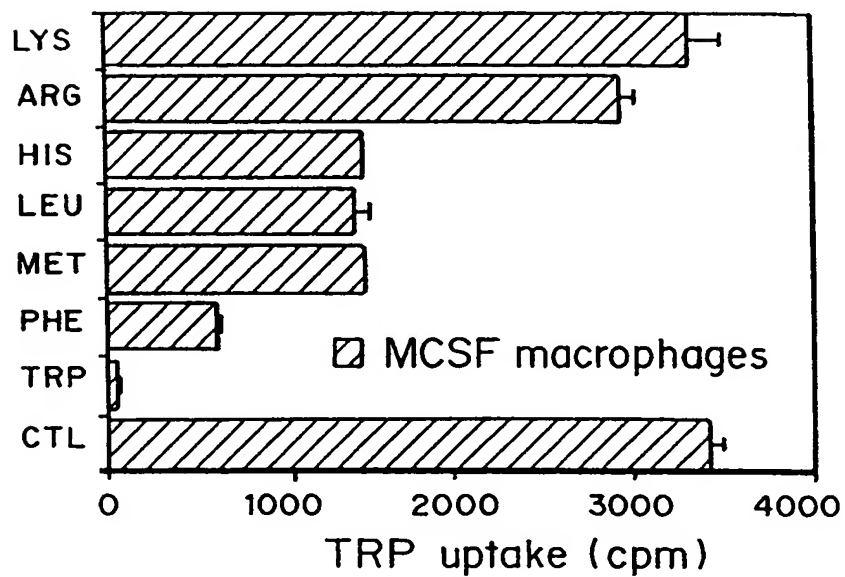


FIG. 8B

6/6

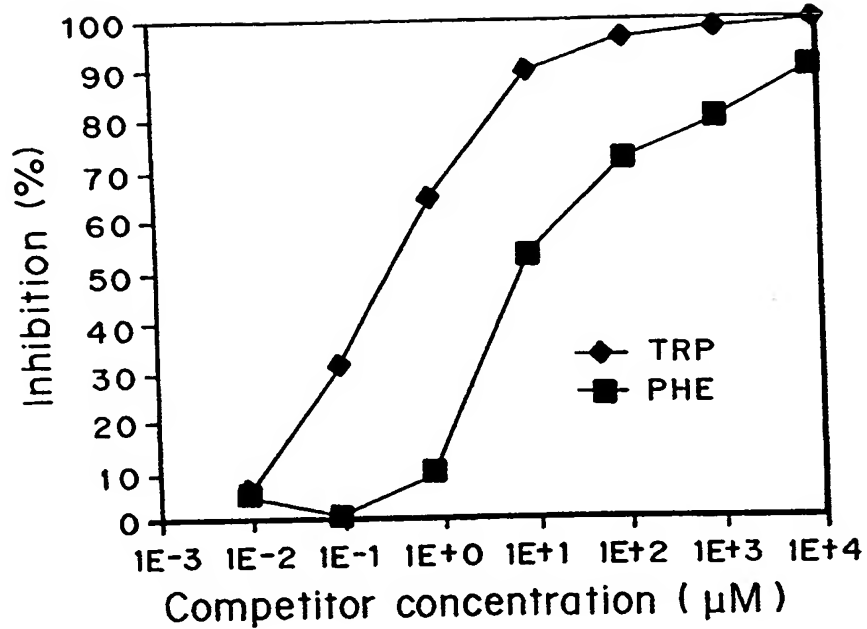


FIG. 9

FIG. 10

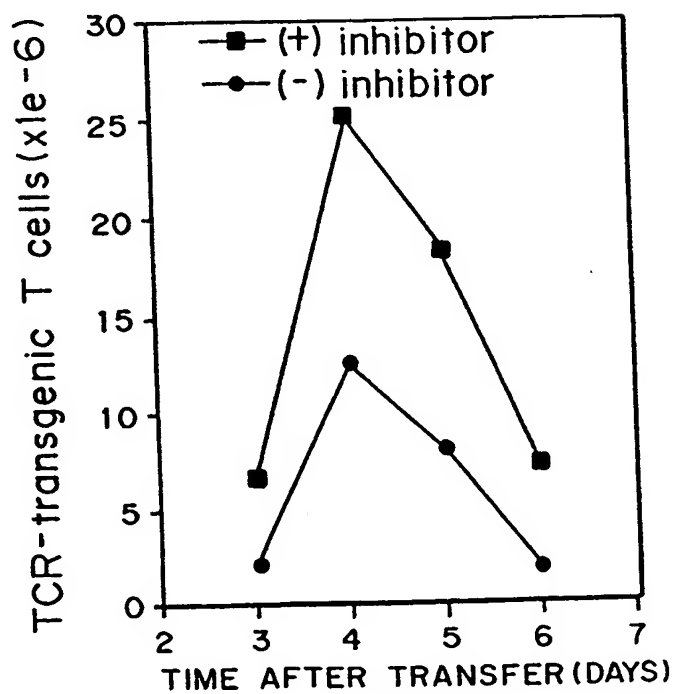
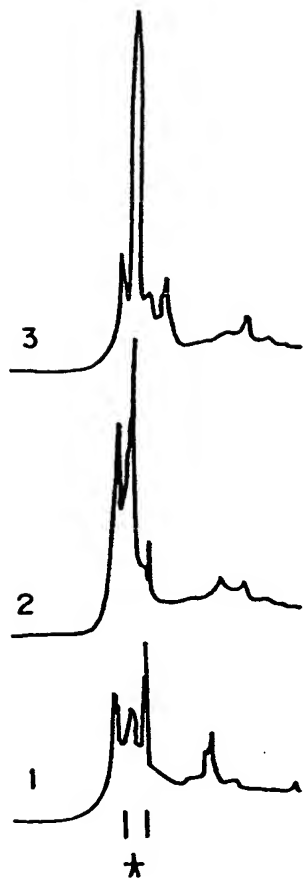


FIG. 11



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 31/405, 31/34, 31/38, 38/41, 38/44, G01N 33/48	A3	(11) International Publication Number: WO 99/29310 (43) International Publication Date: 17 June 1999 (17.06.99)
(21) International Application Number: PCT/US98/25840 (22) International Filing Date: 4 December 1998 (04.12.98) (30) Priority Data: 60/067,610 5 December 1997 (05.12.97) US 60/080,380 1 April 1998 (01.04.98) US 60/080,384 1 April 1998 (01.04.98) US (71) Applicant: MEDICAL COLLEGE OF GEORGIA RE- SEARCH INSTITUTE, INC. [US/US]; 1120 15th Street, Augusta, GA 30912-4810 (US). (72) Inventors: MUNN, David; 967 Meigs Street, Augusta, GA 30904 (US). MELLOR, Andrew; 480 Falcon Drive, Augusta, GA 30907 (US). (74) Agent: PABST, Patrea, L.; Amall Golden & Gregory, LLP, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GA 30309-3450 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims</i> <i>and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 6 January 2000 (06.01.00)
(54) Title: REGULATION OF T CELL-MEDIATED IMMUNITY BY TRYPTOPHAN AND ITS ANALOGS (57) Abstract A mechanism of macrophage-induced T cell suppression is the selective elimination of tryptophan and/or increase in one or more tryptophan metabolites within the local macrophage microenvironment. Studies demonstrate that expression of IDO (indoleamine 2,3-dioxygenase) can serve as a marker of suppression of T cell activation, and may play a significant role in allogeneic pregnancy and therefore other types of transplantation, and that inhibitors of IDO can be used to activate T cells and therefore enhance T cell activation when the T cells are suppressed by pregnancy, malignancy or a virus such as HIV. Inhibiting tryptophan degradation (and thereby increasing tryptophan concentration while decreasing tryptophan metabolite concentration), or supplementing tryptophan concentration, can therefore be used in addition to, or in place of, inhibitors of IDO. In particular, the inhibitor is selected from the group consisting of 1-methyl-DL-tryptophan, β -(3-benzofuranyl)-DL-alanine, β -[3-benzo(b)thienyl]-DL-alanine, and 6-nitro-L-tryptophan. Similarly, increasing tryptophan degradation (thereby, decreasing tryptophan concentration and increasing tryptophan metabolite concentration), for example, by increasing IDO concentration or IDO activity, can suppress T cells. Although described particularly with reference to IDO regulation, one can instead manipulate local tryptophan concentrations, and/or modulate the activity of the high affinity tryptophan transporter, and/or administer other tryptophan degrading enzymes. Regulation can be further manipulated using cytokines such as macrophage colony stimulating factor, interferon gamma, alone or in combination with antigen or other cytokines.		

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INTERNATIONAL SEARCH REPORT

Int. l. Application No
PCT/US 98/25840

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 A61K31/405 A61K31/34 A61K31/38 A61K38/41 A61K38/44 G01N33/48		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 A61K G01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BLIZNAKOV, EMILE G.: "Serotonin and its precursors as modulators of the immunological responsiveness in mice" J. MED. (WESTBURY, N. Y.) (1980), 11(2-3), 81-105 CODEN: JNMDB0; ISSN: 0025-7850, XP002106391 see abstract see page 82, paragraph 2 - page 83, paragraph 3 see page 86, paragraph 1 - page 99, paragraph 2; figures 1-7,9-11 <div style="text-align: center;">--- -/--</div>	1,2,4,5, 8,9,11, 14, 16-18, 26,27
<div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div>		
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Date of the actual completion of the international search <div style="text-align: center;">28 June 1999</div>		Date of mailing of the international search report <div style="text-align: center;">16. 11. 1999</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer <div style="text-align: center;">A. Jakobs</div>

INTERNATIONAL SEARCH REPORT

Int. l. Application No.

PCT/US 98/25840

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HAYAISHI O.: "Utilisation of superoxide anion by indoleamine oxygenase-catalyzed tryptophan and indoleamine oxidation" ADV. EXP. MED. BIOL., vol. 398, 1996, pages 285-289, XP002106392 see abstract see page 287, paragraph 4 - page 288, paragraph 6	1-3,5,8, 9,17-22, 26,28
X	--- TAKIKAWA ET AL.: "Mechanism of Interferon-gamma Action" J. BIOL. CHEM., vol. 263, no. 4, 1988, pages 2041-2048, XP002106393 see abstract see results	1-5, 9-11,17, 18,26,28
X	--- SOUTHAN ET AL.: "Structural requirements of the competitive binding site of recombinant human indoleamine 2,3-dioxygenase" MED. CHEM. RES., vol. 6, no. 5, 1996, pages 343-352, XP002106394 see abstract see page 344, paragraph 1 - page 334, paragraph 3; figures 1,2; table 1 see page 347, paragraph 1 - page 350, paragraph 2	1-4,9, 10, 14-18, 26-30
X	--- PETERSON ET AL.: "Evaluation of functionalized tryptophan derivatives and related compounds as competitive inhibitors of indoleamine 2,3-dioxygenase" MED. CHEM. RES., vol. 3, no. 8, 1994, pages 531-544, XP002106395 see abstract; tables 1,2 see page 532, paragraph 1 - page 539, paragraph 2	1-4,9, 10, 15-18, 26-30
X	--- CADY ET AL.: "1-Methyl-DL-tryptophan, beta-(3-benzofuranyl)-DL-alanine (the oxygen analog of tryptophan), and beta-[3-benzo(b)thienyl]-DL-alanine (the sulfur analog of tryptophan) are competitive inhibitors for indoleamine 2,3-dioxygenase" ARCH. BIOCHEM. BIOPHYS., vol. 291, no. 2, 1991, pages 326-333, XP002106396 see discussion see abstract	1-4, 26-30
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/25840

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KAMATH, SAVITRI K. ET AL: "Amino acid-restricted diets in the treatment of mammary adenocarcinoma in mice" J. NUTR. (1988), 118(9), 1137-42 CODEN: JONUAI;ISSN: 0022-3166, XP002106397 see abstract	1-3,5,9, 10,17,18
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X	--- SIDRANSKY, HERSCHEL ET AL: "Effect of tryptophan on hepatoma and host liver of rats. Influence after treatment with hypertonic sodium chloride and carbon tetrachloride" EXP. MOL. PATHOL. (1981), 35(1), 124-36 CODEN: EXMPA6;ISSN: 0014-4800, XP002106400 see abstract see page 130, paragraph 3 - page 133, paragraph 1; table 4	1,2,4,5, 8-10, 16-18, 26,28
A	--- SCHROEDER, HEINZ C. ET AL: "Suppression of the modulatory effects of the antileukemic and anti-human immunodeficiency virus compound avarol on gene expression by tryptophan" CANCER RES. (1989), 49(8), 2069-76 CODEN: CNREA8;ISSN: 0008-5472, XP002106401 see the whole document	1,26-30
X	--- EP 0 385 385 A (MORINAGA MILK INDUSTRY CO LTD) 5 September 1990 see abstract	1,19,20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/25840

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-25 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

see additional sheet, subject 1.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Further defect(s) under Article 17(2)(a):

In view of the large number of compounds, which are defined by the general definition(s)/formulae used in claims 1-29,31-38 the search had to be restricted. the search was limited to the compounds for which pharmacological data was given and/or the comounds mentioned in the claims, and to the general idea underlying the application.

Regarding claim 15: "inhibiting tryptophan decregation" was considered to be an obvious error and searched as meaning "inhibiting tryptophan degradation".

Claim 33 is missing.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-22,26-29,35-38 (all partially), 30

Use of 1-methyl-DL-tryptophan, beta-(3-benzofuranyl)-DL-alanine, beta-[3-benzo(b)thienyl]-DL-alanine, and 6-nitro-L-tryptophane as immunomodulators, to regulate rejection or acceptance of allograft or xenograft, to prevent, terminate or promote pregnancy, to induce rejection of cells, to reverse the immunosuppressed state found in AIDS and to decrease an autoimmune response.

2. Claims: 1-22,26-28,31,35-38 (all partially), 32

Use of recombinant IDO (indoleamine-2,3-dioxygenase) as immunomodulator, to regulate rejection or acceptance of allograft or xenograft, to prevent, terminate or promote pregnancy, to induce rejection of cells, to reverse the immunosuppressed state found in AIDS and to decrease an autoimmune response.

3. Claims: 1-22,26-28,31,35-38 (all partially), 32

Use of indolyl-3-alkane alpha-hydroxylase as immunomodulator, to regulate rejection or acceptance of allograft or xenograft, to prevent, terminate or promote pregnancy, to induce rejection of cells, to reverse the immunosuppressed state found in AIDS and to decrease an autoimmune response.

4. Claims: 1-22,26-28,31,32,35-38 (all partially), 34

Use of an inhibitor of the high affinity, sodium independent tryptophan transport system as immunomodulators, to regulate rejection or acceptance of allograft or xenograft, to prevent, terminate or promote pregnancy, to induce rejection of cells, to reverse the immunosuppressed state found in AIDS and to decrease an autoimmune response.

5. Claims: 23-25

A method for assessing the immunological state of cells

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/25840

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0385385 A	05-09-1990	JP 2225418 A	07-09-1990
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